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# *The Journal of Infectious Diseases*

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**THE  
JOURNAL OF INFECTIOUS DISEASES**



# *The* Journal of Infectious Diseases

Published by the Memorial Institute for Infectious Diseases

EDITED BY

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IN CONJUNCTION WITH

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No. 1

## ON THE OCCURRENCE OF AN ISOLATED ANTIBODY IN THE CEREBROSPINAL FLUID.\*

LUDVIG HEKTOEN.

(From the Memorial Institute for Infectious Diseases, Chicago.)

In the work by Dr. Carlson and myself on the distribution of antibodies in the body fluids of dogs,<sup>†</sup> we found that in dogs, injected with rat blood, opsonin for rat corpuscles appeared in the cerebrospinal fluid, which, however, did not contain a trace of agglutinin. In the serum of the blood and the lymph, both these antibodies described parallel curves. The curve of the opsonin concentration in the cerebrospinal fluid described exactly the same course as in the blood and lymph, but remained much lower. In Chart 1 are reproduced composite curves showing these relations and obtained from estimations on dogs killed at varying intervals after the intravenous injection of one cubic centimeter of 10 per cent suspension of rat blood per kilo of the weight of dog.

In dogs injected intravenously with goat blood we did not find any agglutinin in the cerebrospinal fluid. During the period of highest antibody content in the blood and lymph, however, the fluid contained traces of lysin and opsonin.

\* Received for publication November 15, 1912.

† *Jour. Infect. Dis.*, 1910, 7, p. 319.

The fact that opsonin and not agglutinin appeared in the cerebrospinal fluid of dogs injected with rat blood was interesting because it seemed to point to the separate entity of these antibodies. There was a possibility, however, that agglutination and opsonification might be caused by one substance, the agglutinative action of which was suppressed by the influence of the cerebrospinal fluid. Consequently, on account of the theoretical importance of the question of the separate entities of the bodies concerned in the various forms of antibody action, it seemed desirable to study the antibody

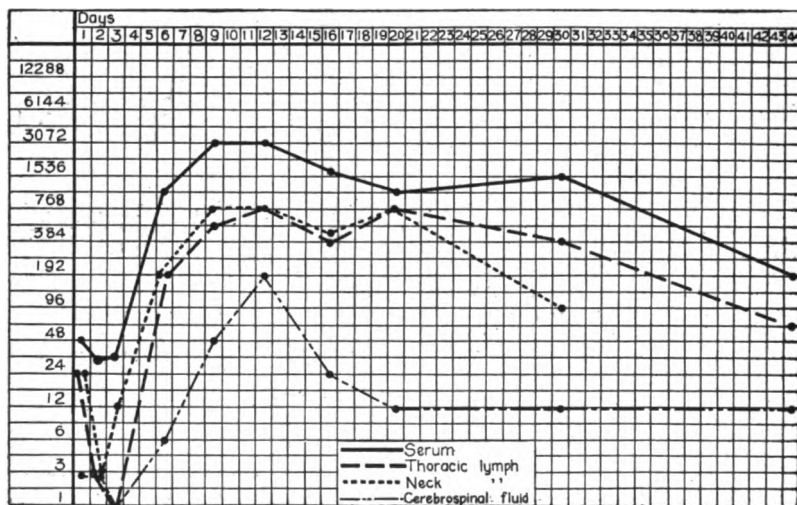


CHART 1.—Specific opsonin in blood, lymph, and cerebrospinal fluid of dogs injected with rat blood.

content of the cerebrospinal fluid of dogs a little more closely. In this article is given a brief account of certain further observations.

The methods used to determine the antibody content of various fluids are the same as were used by Dr. Carlson and myself. The cerebrospinal fluids used in these experiments were obtained without any admixture whatsoever of blood, and they appeared absolutely clear. In no case were there any inflammatory or other changes in the membranes and other structures of the central nervous system.

1. Does the cerebrospinal fluid inhibit agglutination of rat corpuscles?

I have found that in mixtures of normal and immune dog serum and rat corpuscles, the replacement of the salt solution with cerebrospinal fluid of normal dogs in no way diminishes the agglutinative action of the serum.

Several dogs, given a single intravenous injection of rat blood, one cubic centimeter of a 10 per cent suspension per kilo of weight, have been killed at varying intervals and bled dry so that the cerebrospinal fluid could be withdrawn easily without the slightest admixture of blood. This quantity of rat blood injected intravenously causes an abundant production of antibodies and may be regarded as an optimum antigenic dose for the dog.

The serum of one such dog (223) killed on the eighth day after injection, agglutinated and opsonized rat corpuscles in a dilution of 1 to 768. The cerebrospinal fluid did not agglutinate the corpuscles but it had a distinct opsonic effect at 1 to 96. The addition of this fluid to the serum of this dog and to normal dog serum in place of salt solution in preparing dilutions of varying strengths, and allowing the mixture to remain at 37° C. for two to three hours, did not reduce the agglutinative power of the sera at all. The same results were obtained with the cerebrospinal fluids from other dogs (187, 232, 233) in both earlier and later stages of antibody formation, the opsonic value of the fluid varying from 1-24 to 1-384.

From these results it may be concluded that the cerebrospinal fluid of dogs, whether normal or immunized with rat blood, does not inhibit the agglutinative action of dog serum on rat corpuscles.

2. Is the opsonic action of the cerebrospinal fluid of dogs injected with rat blood due to the same substance as the opsonin in the blood and lymph?

The indications all point to the presence in the cerebrospinal fluid of the same opsonin as in the blood and lymph.

In the first place the concentration though much less than in the blood and lymph describes the same general course as shown in Chart 1. Other estimations of the opsonin content of the blood and cerebrospinal fluid of immunized dogs, the two estimations in each case being made under exactly comparable conditions, give the following results:

Blood	Cerebrospinal Fluid	Blood	Cerebrospinal Fluid
6,144.....	384	768.....	48
768.....	24	1,536.....	96
1,536.....	48	1,536.....	96

In every case the agglutinin content of the blood corresponded closely to the opsonin content while the cerebrospinal fluid was devoid of all agglutinative effect. Furthermore, the opsonin in the cerebrospinal fluid is specific for rat corpuscles to which it becomes bound so that the fluid loses opsonic power after being treated for an hour or two with a sufficient amount of corpuscles and then centrifuged clear.

The opsonic power of the fresh fluid of dogs injected with rat corpuscles does not seem to be diminished by heating the fluid to 58° C. for 30 minutes. Inasmuch as the opsonic power is intensified by the addition of normal dog serum in quantities so small as to have no demonstrable opsonic action by themselves (Table 1), it may be concluded that the fluid contains a specific, thermostable opsonin which cannot be differentiated from that in the immune serum, that is, in the blood and lymph.

TABLE 1.  
REACTIVATION OF THE OPSONIN IN CEREBROSPINAL FLUID.

Cerebrospinal Fluid of Immunized Dog	Fresh Dog Serum	Phagocytosis of Rat Corpuscles
.025 c.c.	.....	0
.025 c.c.	.005 c.c.	+
.025 c.c.	.0025 c.c.	+
.....	.005 c.c.	0

3. Are lysin, precipitin, and complement-deviating antibodies present in the cerebrospinal fluid of dogs injected with rat blood?

I have not been able to obtain any evidence that the cerebrospinal fluid of dogs immunized with rat blood contains any lysin for rat corpuscles. Lysis does not occur in mixtures of rat corpuscles and cerebrospinal fluid to which complement is added in the form of guinea-pig serum. As rat corpuscles do not seem to be laked easily by dog lysin it is of course possible that the fluid may contain lysin in quantities so small as to escape detection by the usual method.

Neither has it been possible to detect the presence of any complement binding substances in the cerebrospinal fluid of dogs injected with rat blood and the serum of which, withdrawn at the same time as the fluids, readily bound the complement in guinea-pig serum in mixtures with rat corpuscles. The indicator used was antsheep rabbit serum and sheep corpuscles. The results obtained are illustrated by the experiment given in Table 2.

Finally, precipitin for rat serum has not been demonstrated in the cerebrospinal fluid even when the corresponding dog serum (262) caused a precipitate to form in rat serum diluted 1:1280.

TABLE 2.  
EXPERIMENT ON COMPLEMENT-DEVIATION BY SERUM AND CEREBROSPINAL FLUID OF DOG INJECTED WITH RAT BLOOD.

ANTIGEN (5 PER CENT RAT BLOOD)	SERUM OR CEREBROSPINAL FLUID OF DOG INJECTED WITH RAT BLOOD	COMPLEMENT (GUINEA-PIG SERUM)	HEMOLYTIC SYSTEM		LYSIS
			Antisheep Amboceptor	Sheep Corpuscles 5 Per Cent	
0.2 c.c.	Serum 0.05 c.c.	0.0125 c.c.	0.007 c.c.	0.5 c.c.	0
"	" 0.003 "	"	"	"	+++
"	Fluid 0.15 "	"	"	"	+++
"	" 0.1 "	"	"	"	+++
.....	.....	"	"	"	+++
	Serum 0.5 "	"	"	"	+++

The serum agglutinated and opsonized rat corpuscles at a dilution of 1:1536.

The cerebrospinal fluid opsonized rat corpuscles at a dilution of 1:96.

4. Does opsonin enter the cerebrospinal fluid after passive immunization with respect to rat blood?

So far as the antibodies for goat blood are concerned Dr. Carlson and I found the distribution after passive immunization by transfusion to correspond closely to the distribution in actively immunized animals. We made no observations, however, on the distribution of antibodies for rat corpuscles after passive immunization. I have found that in dogs injected with antirat dog serum the cerebrospinal fluid acquires some opsonic but no agglutinative power with respect to rat corpuscles. The observations in one case were made twenty-four hours after the intracardiac injection of the immune serum, and at this time the serum of the injected animal agglutinated rat corpuscles in a dilution of 1:768 and opsonified at 1:384, while the cerebrospinal fluid was opsonic at 1:6.



## SUMMARY.

It appears that the cerebrospinal fluid of the dog does not inhibit the agglutination of rat corpuscles by the serum of this animal; that the opsonic effect of cerebrospinal fluid of dogs injected with rat blood is due to a specific, thermostable opsonic substance, seemingly identical with that in the blood and lymph, this being the only antibody demonstrable in the cerebrospinal fluid of dogs so immunized; and finally that this opsonin makes its way into the fluid after passive immunization.

These results indicate that opsonin can occur as a distinct substance and that its presence in the cerebrospinal fluid is the outcome of some form of selective process.

## THE LABORATORY DIAGNOSIS OF GLANDERS.\*

E. MARION WADE.

(From the Bacteriological Laboratory of the Boston Board of Health.)

Glanders, although one of the earliest known diseases of horses, was first considered infectious in the 17th century, and toward the end of the 18th Abildgaard and Viborg proved it to be so. This was later disputed by various authorities, until Rayer in 1837 demonstrated its transmissibility to man and Chaveau in 1868 showed that the virus was contained in the lesions of the affected animal. Even after the infectious nature of the disease was determined, many varying opinions were held as to the causative agent, until in 1882 Loeffler and Shutz settled all disputes by finding an organism which fulfilled all of Koch's postulates. While these discoveries meant much, still the bacteriological diagnosis had been very little aided, as the isolation of the specific organism by plating from lesions or nasal discharges is a long and tedious process and is met with very little success, as will be shown later. In 1886 Strauss proposed the intraperitoneal inoculation of male guinea-pigs with the suspected material as a quick means of diagnosis. Usually within three to four days the scrotum becomes red and shining and suppuration occurs. This pus contains the specific bacillus usually in pure culture.

Mallein, a toxin of the glanders bacillus, was first discovered by Helman and Kalning in 1891. They used filtered aqueous and glycerin extracts of potato cultures. Roux cultivated *B. mallei* in flasks of five per cent glycerin broth. This latter is the method generally used. The cultures are grown for six weeks in some laboratories, for five months in others. This is then boiled to kill the organisms and concentrated to one-half its volume, filtered through a Berkefeld candle and diluted with an equal volume of one per cent carbolic acid, making the final product 0.5 per cent carbolic acid. The diagnostic dose is 2 c.c. At the present time the ophthalmic test is being tried, using a concentrated extract,

\* Received for publication November 1, 1912.

and is meeting with encouraging results. Although some work had been done with the agglutination tests by McFadyean and others and Pfeiler and Konew had used the precipitation test, the next marked advance in the laboratory diagnosis was made in 1910 when Schutz and Schubert applied the complement-fixation test to glanders. This test supplemented by the agglutination test is at the present time taking the leading place in the diagnosis of glanders by public health laboratories and cattle bureaus.

The two most essential factors in all laboratory diagnoses are accuracy and early reports, and a comparison of the methods in use for glanders along this line is interesting. In a series of 1,600 examinations by the use of guinea-pigs after the Strauss method covering a period of six and one-half years 497 or 31.16 per cent gave a positive result. Of these positive cases the cultures made direct from the suspected material on potato gave a characteristic growth in only 62 instances or in 12.5 per cent. The remainder of the cultures were overgrown either with mold or other organisms and any attempts to isolate *B. mallei* would have been unsuccessful. Results obtained from a small series of such attempts seem to justify this statement. In 24 of the 62 cases the inoculated guinea-pigs had shown no lesions up to the time of development of a characteristic growth on the culture and the same pigs were reinoculated with an aqueous suspension of the culture and developed typical scrotal lesions on the second or third day. In these cases therefore the culture made direct from the suspected material enabled the giving of a positive report which might otherwise have been missed. In the remaining 38 cases the pigs showed lesions at the time of the characteristic growth on potato. This leaves 435 out of the total 497 in which the culture direct was of no assistance although 14 times very suspicious organisms had been found in the smear made from the material. Before a final positive report is given by this method three things are essential—typical lesion in the scrotum of the guinea-pig, typical organisms in the smear from the pus at the time of autopsy of the pig, and a characteristic growth on potato from the lesion in the pig. In several instances a similar lesion has been produced by other organisms—*B. pyocyaneus* being the most common. This can be readily distinguished

from *B. mallei* by its much darker color on potato and its more moist and abundant growth often extending around the whole surface of the medium.

Varying opinions are held as to the length of time that *B. mallei* will retain its virulence upon artificial media without passage through animals—from two or three generations to at least two months. Ten strains isolated between September 23 and October 31, 1911, have been twice passed through guinea-pigs—once November 29, 1911, and the second time March 4, 1912. Four of these cultures were inoculated September 6, 1912, after being on artificial media six months, and in each case the pig developed a typical Strauss reaction on the second day, as had been the case in the previous inoculations. From this it is evident that at least some strains of *B. mallei* retain their virulence on artificial media for six months. These same cultures will be kept and tested periodically. Much still remains to be done on the length of life of the organism in a dried condition as met with in infected stables, on the tie posts and halters, and the chances of conveying infection from horse to horse by the walls and tie chains in blacksmith shops, not to mention the public watering trough.

The diagnosis of glanders by the precipitation test recommended by Pfeiler in Germany and Konew in Russia has not met with general corroboration. In a few cases tried by the writer the results were not found to agree with the diagnoses by other methods.

The agglutination test for glanders was first suggested in 1896 by McFadyean after Widal's test had been adopted for typhoid fever. It was not extensively used, however, until Schutz and Meissner established its value in 1905. Since this time the test has been widely employed but it is not absolutely reliable as by it alone some cases would be missed. Whether or not it is more reliable in acute and less so in chronic cases has yet to be proved. The macroscopic agglutination test with the aid of the centrifuge seems to be the most reliable of the agglutination tests. To make the test fluid pure cultures of several strains of *B. mallei* are grown on two and one-half per cent glycerin agar in flasks or, as is more commonly the case, in wide-mouth bottles (the media covering one

of the large surfaces), in order to get a large area for growth. After 24 to 36 hours in the incubator at 37° C. the cultures are killed by heating to 60° C. for two hours and then washed off the surface of the media with physiological salt solution containing 0.5 per cent carbolic acid. The fluid is filtered through ordinary filter paper and diluted to the proper density by comparison with a known agglutination fluid and by testing against sera of known agglutination power. The fluid should not be tested until it has stood at ice-chest temperature for eight to 10 days. It keeps well for two to three months.

In making the test a basic dilution of the serum to be examined is made and from this other dilutions can be prepared. A dilution of 1:40 has been recommended for the basic dilution but the writer has used 1:80 in order to lessen the possibility of error in the high dilutions where 0.01 c.c. would be used. Varying amounts of the basic dilution are placed in tubes according to the table below.

1:600	by using	.26	c.c.	of basic dilution.
1:800	" "	.20	" "	" "
1:1000	" "	.16	" "	" "
1:1200	" "	.14	" "	" "
1:1400	" "	.12	" "	" "
1:2000	" "	.08	" "	" "
1:4000	" "	.04	" "	" "
1:8000	" "	.02	" "	" "

To each of these tubes two cubic centimeters of agglutination fluid is added. Two control sera—a known positive and a known negative—are run with each set of tests. The tubes are placed in the incubator at 37° C. for a half hour, then centrifugalized for 10 minutes at a speed of 1,600 revolutions a minute. The readings are made after the tubes have stood at room temperature for at least two hours, and if there is no immediate hurry for a report it is better to let them stand over night. The readings can best be made by looking down through the tubes against a dark surface. In a positive reaction the bacteria will be seen in clumps and spread over the bottom of the tube, but in a negative case there will be a round, dense-white precipitation in the center of the bottom of the tube. There is so much variation in the agglutinating power of normal serum that a positive report has not been given unless an

agglutination has been obtained at a dilution of 1:2000. If there has been an agglutination at 1:1400 an atypical report has been given, and other specimens of blood asked for. The dividing line between a positive and a negative report should be established by the laboratory making the tests, as different agglutination fluids made by different workers seem to differ although of the same apparent density. The fluid in use in the tests reported below has been made from eleven strains of *B. mallei* isolated in September and October, 1911.

In the tests by complement fixation the technic followed has been that recommended by Mohler and Eichhorn,<sup>2</sup> with a few modifications. This differs from the technic usually employed in the Wassermann test mainly in the titration of the complement and using the smallest amount which with two units of amboceptor gives complete hemolysis instead of using always a 10 per cent solution and varying the amount of amboceptor used and thus having an excess of complement.

In the preparation of the amboceptor it has been found better to lessen the time between the inoculations, thereby lessening the chance of losing the rabbit by anaphylaxis. An amboceptor titrating 1:2000 was obtained in one instance by giving inoculations of 5, 10, and 15 c.c. respectively on three successive days and bleeding on the ninth day after the last inoculation. This amboceptor retained a strength of 1:1500 until it was all used six months later. Reinoculations of 5, 10, and 15 c.c. were made into the same rabbit at three-day intervals but it died on the second day after the last inoculation although appearing to be in perfect health on the night previous.

The glanders antigen has been the one troublesome feature in the test, especially because of its instability. Also some clinical cases of glanders on which guinea-pigs have given a positive result and of sufficient duration to lead to the expectancy of a positive reaction to complement fixation have failed to give it. Most of the antigens in use at the present time in this country are shaken but in Germany the shaking is being dispensed with. Syphilitic antigen is being prepared without shaking, as is also gonococcus

<sup>2</sup> *Bulletin* 136, Bureau of Animal Industry, Washington, D.C.

antigen, and many working with complement-fixation tests here are beginning to follow the example of the Germans. For this reason it has been thought advisable to try some glanders antigens in the same way. The first attempt was to grow the cultures on neutral veal agar and the growth was very scant and somewhat discouraging, but it was thought that a better growth might be obtained after several transplants had been made on this medium. The growth was very little better but an antigen was made. This was unsatisfactory. Next neutral glycerin agar was used and the growth was also scanty, but an antigen made in the same manner gave a titre of 1:10. The glanders bacillus prefers slightly acid media to grow upon so the ordinary glycerin agar one per cent acid was next used, this being the medium on which the cultures had been growing since their isolation. The antigen made in the same way from this medium yielded a titre of 1:300. The following technic was employed in making the antigen: Eleven strains of *B. mallei* were used, the growth being washed off the surface of the media with sterile distilled water to give a milky suspension. This was then autolyzed for one-half hour at 56° C., after which it was filtered through a Berkefeld candle. This filtrate was then sterilized by heating to 56° C. for two hours on three successive days. For use the antigen is diluted with nine per cent salt solution to make a product 0.9 per cent saline (0.9 c.c. antigen + 0.1 c.c. nine per cent salt solution), otherwise hemolysis might be produced. This antigen has been used in a duplicate series of 117 sera with absolutely reliable results. The ease with which the antigen is prepared and the saving of time recommend its adoption.

One other modification in technic has been tried with very satisfactory results. In the second set of tubes in each test a double quantity of antigen has been used and in the control tube 0.4 c.c. of serum instead of 0.2 c.c. When the control tube containing 0.4 c.c. of serum has completely hemolyzed the readings can be made, as no inhibitory action could then be due to 0.2 c.c. serum. It has been found in many instances that a much sharper reaction has been obtained by using this double quantity of antigen with the 0.2 c.c. serum and the reading changed from a = to a + or even a ++.

During the past year 456 sera have been tested by both complement fixation and agglutination and the results have agreed in 373 instances or almost 82 per cent. The results on the remaining 84 sera from 76 cases are interesting, as will be seen from the following table.

TABLE 1.

	NUMBER OF CASES	FINAL OUTCOME		
		+	Questionable	-
C.F.+ Ag. atyp. ....	6	6	...	...
C.F. atyp. Ag.+ ....	10	8	...	2
C.F.- Ag.+ ....	36	21	4	11
C.F.+ Ag.- ....	5	3	...	2
C.F. atyp. Ag.- ....	3	2	1	...
C.F.- Ag. atyp. ....	10	...	...	10
Total .....	76	40	5	31

The two cases listed as "negative" with C.F. atyp. Ag.+ are from the same stable, neither showing symptoms seven months later—the mate of one had been killed as glandered and from the other an atypical mallein reaction had been obtained. Of those with C.F.-Ag.+ in three cases the agglutination test was made after the horses had been released on the negative complement-fixation report and had changed owners, so no subsequent history has been obtainable, but the veterinarian reports that they were probably glandered horses. In the fourth case blood taken two weeks later gave a positive result by complement-fixation and also by agglutination, but the horse is still living seven months later and shows no symptoms. These four are therefore put in the questionable classification. Ten of the cases called negative were from the routine taking of specimens from stables in which glanders existed, but the horses show no symptoms to date. In the other case the horse was killed but at autopsy no glanders lesions were present—the mate of this horse was glandered.

The two negative cases with C.F.+ Ag.- were also from the routine taking of specimens from a stable and still show no symptoms. The one questionable case with C.F. atyp. Ag.- is at present under observation. Of course some of the cases called negative may have glanders in a latent form, but as no evidence of



it exists at the present time it seems fair to class them as negative as horse owners object to losing horses which show absolutely no external signs and are in apparent good health. This summary of cases seems to indicate that a positive report by complement-fixation or agglutination is strong evidence of glanders while an atypical agglutination without some fixation of complement should not be so considered.

In 135 cases guinea-pig tests have been made as well as blood tests and Table 2 shows the results of these tests.

TABLE 2.  
SUMMARY OF CASES ON WHICH BOTH STRAUSS AND BLOOD TESTS WERE MADE

Positive Cases		Negative Cases	
Both +	56	Both -	32
Blood + Strauss -	30	Blood - Strauss Uns.	5
Blood + Strauss Uns.	10		
Blood - Strauss +	2		37
	<hr/> 98		

This shows that in 45 instances out of 135 or 33.3 per cent the pigs failed to give a reliable diagnosis and two cases in which the blood test failed but in one of these instances the agglutination test was not made.

#### CONCLUSIONS.

1. The diagnosis of glanders by the complement-fixation test supplemented by the agglutination test on all negative sera is the quickest and most reliable test at the present time.
2. Glanders antigen prepared without shaking but filtered through a Berkefeld candle gives reliable results and yields a more stable product.
3. Normal horse serum varies widely in its agglutinating power, therefore a weak positive should be considered in conjunction with the complement-fixation test or with clinical symptoms.
4. Antigens and agglutination fluids should be prepared from several strains of *B. mallei*.
5. While a positive result from guinea-pig inoculation is conclusive evidence of the presence of glanders, failure of the pigs to develop lesions is not proof of its absence.

## TWO LINCOLN (NEBRASKA) TYPHOID FEVER EPIDEMICS OF 1911 AND 1912.\*

HERBERT H. WAITE.

*(From the Bacteriological Laboratory, University of Nebraska, Lincoln, Nebraska.)*

On August 29, 1912, I was requested by the Board of Health of the city of Lincoln to make an investigation to determine if possible the origin and cause of an outbreak of a disease reported as typhoid fever which was then prevailing. This outbreak was said to be most prevalent in one particular portion of the city and consequently attention was turned to that portion. Of the cases reported an investigation was made of over 60 individuals and after considering all the probable causes for the majority of those affected the conclusion was reached that all could be logically excluded except water.

### DESCRIPTION OF CITY WELLS.

The city of Lincoln has a population of about 45,000. The city water supply is owned and controlled by the city. At the time of both typhoid epidemics the city was supplied by the combined output of the three wells described below. The average daily supply from these three wells was about 3,275,000 gallons. Of this amount the A Street well delivered about 2,500,000 gallons, the Rice well 600,000 gallons, the F Street well 175,000 gallons. To produce this output required continuous pumping for 24 hours daily at the A Street well, from six to eight hours daily at the F Street well, and 20 hours daily at the Rice well. At the present time, August 22, 1912, the combined capacity of all the city wells, provided they were all used 24 hours per day, would be 7,000,000 gallons. The increase in the amount of water which could be obtained has been secured through the construction of five new driven wells. These new wells have water-tight casings from the water-bearing strata to the surface. Their combined capacity is 4,500,000 gallons per day. The remaining 2,500,000 gallons represent the combined

\* Received for publication September 5, 1912.

capacity of the A and F Street wells, the Rice well having been permanently abandoned.

#### THE MOCKETT OR A STREET WELL.

[In describing the city wells I shall quote directly from the report of Mr. James C. Harding, one of the associates of Mr. George W. Fuller, consulting hydraulic engineer and sanitary expert.]

"The A Street well, pumping-station, and reservoirs are located on A Street between the C.R.I. & P. Railroad and Normal Boulevard. The main well is circular, about 47 feet in diameter at the top and for a distance of about 28 feet below the surface of the ground. There it narrows to about 37 feet for a distance of 27 feet. The bottom section, 33 feet 7 inches in diameter, is about 22 feet deep, making a total depth to the sandstone of about 77 feet. The well casing is of brick masonry 16 inches thick in the two upper sections and 12 inches thick in the lower. Up to a few years ago the well was uncovered, but has been recently roofed over with a corrugated steel roof. In the bottom of this well are six borings made to a depth of from 50 to 100 feet in the sandstone, and it is through these borings that practically all the water is obtained.

"Besides the main well there are six eight-inch driven wells operated by air lifts which discharge water directly into the storage reservoirs.

"*Water levels.*—The normal water level in the main well when it was first built was about 30 feet below the surface of the ground when the pumps were not operating. At the end of a day's pumping when the water was being taken from the well at an average rate of 2,000,000 gallons per day the water level was about 60 feet below the surface of the ground. At the present time with the Rice well out of commission and with the pumps taking water as fast as it flows into the well, the sandstone at the bottom is exposed during the pumping and the water level stands only about 75 feet below the surface of the ground. From records obtained by the city engineer it is evident that the water level around this well has been lowered during the last 10 years about 11 feet.

"*Pumping equipment.*—During normal operation the water from the main well and from the air lifts is discharged into the storage reservoirs and from there pumped to the mains by high-lift pumping engines. The pump has a rated capacity of three million gallons a day. This is the pump which is ordinarily used and which furnishes the main supply for the city.

"*Storage reservoirs.*—On the south side of A Street and east of the pumping-station are located the two covered masonry storage reservoirs. The first of these is about 60 by 190 feet on the inside and has a capacity of about 1.3 million gallons. The second reservoir, built more recently, is about 45 by 190 feet inside and has a capacity of about one million gallons.

"*Geology.*—The record of the different strata encountered in drilling a test well, located about 700 feet west of the A Street pumping-station, as given by the city engineer, shows surface soil to a depth of 2 feet, clay to 20 feet, and sand to 50 feet. The sandstone below this varies in coarseness to 175 feet, and below this depth very fine sandstone to 198 feet. A stiff clay was encountered at 203 feet. The water level was 48 feet below the surface of the ground.

"*Sewers and surroundings.*—There is a 10-inch vitrified sewer running from the alley between A Street and Washington Street and in Washington Street and across

private land from Washington Street to A Street. At one point this sewer is within 20 feet of the southeast corner of the smaller storage reservoir. At the nearest point it is about 200 feet from the main well. The 16-inch discharge line from the air lifts and driven wells crosses this sewer near the reservoirs about 4.5 feet below the invert of the sewer.

"Northeasterly of the main well is the old channel of Antelope Creek. This channel has been filled in at both ends, leaving a hole along the old stream bed at a distance of from 150 to 300 feet from the well. Probably some surface wash from the street collects in this hole and possibly percolates through the ground into it.

"*Quality of the water.*—So far as we know, there have been no chemical analyses made of the water of this well.<sup>1</sup> Dr. Waite has, however, made some bacterial analyses and on some occasions has found results which he believes show that some polluting matter is entering the well on the east side. From the surroundings of the well and from our own investigations we did not see anything to cause us to condemn this well for water supply purposes. There is a small amount of ground water leaking into the well at various places which, according to Dr. Waite, have in some instances shown indication of being contaminated by some foreign matter. Such conditions, however, might easily have arisen from the fact that this seepage is probably surface water and contains matters not filtered out by its passage through the ground.

#### F STREET WELL SUPPLY.

"*Well.*—The F Street well is an open well somewhat similar to that just described, but differing in that it does not extend to the sandstone but rests on a bed of clay overlying the gravel strata. This well is 39 feet in diameter at the top and for a depth of 26 feet below the surface of the ground. Inside of this upper section there is a second well 29 feet in diameter and about 20 feet deep. The walls of this well are of brick masonry, the outside section about two feet thick and the inside about 15 inches. The well is covered by a corrugated steel roof. At the time the well was constructed a hole was dug some 12 feet below the inner well section to gravel, but it was found upon testing the water that it was very salty, and an effort was made more or less successfully to close up this opening and cut out the salt water. As shown by the analysis of the water, however, it is apparent that considerable of this salt water still gets into the well.

"*Pumping equipment.*—This well is equipped with an electrically operated pump. This pump has a capacity of about 1,500,000 gallons per day, but is used only about five hours out of the 24, as there is not sufficient water to operate it for a greater length of time.

"*Geology.*—In sinking this well, soil was encountered to a depth of about 25 feet below the surface. Below this was a gravel layer about two and one-half feet in thickness resting on a bed of yellow clay about eight feet in depth. The bottom of the shoe of the outer section of the well stops at approximately the top of this clay stratum. Below this clay is a gravel layer about two feet in thickness, and below this is blue clay about 13 feet in depth and a third gravel layer of about 10 feet in depth. It is from this gravel that most of the water is obtained. Below this is a four-foot layer of clay and a gravel layer of about the same thickness in which the salt water mentioned above is encountered.

<sup>1</sup> Several chemical analyses of the water from this well had been made previous to this physical examination and could have been easily obtained.

*"Sewers and surroundings.*—On Sixth Street there is a 15-inch sewer which takes the sewage from a large part of the business part of the city and which at times is greatly overloaded, so much so, in fact, that the water is backed up to the surface of the roadway on Sixth Street at times of heavy rainfall. This sewer is about 60 feet from the side of the well. There is also a four-inch tile drain connecting with this 15-inch sewer which passes within eight feet of the well and is connected to the water-closet in the F Street park. This drain was cut off at the curb line in the latter part of January by the city engineer and carefully plugged.

*"Quality of the water.*—While, as stated above, most of the water from this well comes from the lower gravel layers, and, excepting for the large salt content, is apparently of good quality, yet there is every indication that a small quantity of water gets into the well through the upper gravel layer. Whether or not the flow from this place increases greatly during times of high water we do not know, but in all probability it does. We believe that it is reasonable to suppose that the leakage from the sewer on Sixth Street, should it occur, could readily find its way into this well below the outside wall. We have a chemical analysis of the water from this well, made by Mr. C. J. Frankfurter in October, 1911, for Dr. H. H. Waite and taken from his report, as follows:

	Parts per Million	Grains per Gallon
Total solids.....	1,294.6	85.73
Organic and volatile matter.....	111.4	6.52
Albuminoid ammonia.....	.252	.015
Free ammonia.....	.352	.020
Total ammonia.....	.604	.035
Oxygen-consuming power.....	.72	.042
Nitrate nitrogen.....	.65	.038
Nitrite nitrogen.....	None	None
Chlorine.....	545.85	31.93
Sodium chloride (calculated from chlorine content).....	900.00	52.65

"A chemical analysis of water from the F Street well was made on October 10, 1911, by Mr. C. J. Frankfurter. The results of this analysis were as follows:

	Parts per Million	Grains per Gallon
Total solids.....	1,507.5	88.18
Organic and volatile matter.....	142.4	8.33
Albuminoid ammonia.....	.01	.0006
Free ammonia.....	.03	.0017
Total ammonia.....	.04	.0023
Oxygen-consuming power.....	.097	.0056
Nitrate nitrogen.....	2.6	.15
Nitrite nitrogen.....	Trace	Trace
Chlorine.....	606.5	35.48
Sodium chloride (calculated from chlorine content).....	1,000.0	58.5
No charring.....	.....	.....

#### RICE WELL SUPPLY.

*"Well.*—The Rice well, built 23 years ago, is located near N Street between 23d and 24th Streets. The well proper is 24 feet in diameter and about 60 feet deep below

<sup>1</sup> The chemical analysis given above was made from water collected from the Rice well and not from the F Street well. There is no reason why this analysis should have been credited to the F Street well, the record being explicit concerning the Rice well from which the water was obtained.

the surface of the roadway. At the present time the walls of this well are cracked in a number of places, not enough to endanger the structure, but sufficiently to admit of the entrance of water. Besides this there are many places where there is evidence that the ground water has found its way into the well at various times.

*"Water level.*—The elevation of the roadway in front of the well is about 70. The normal water level when not pumping is 32 feet below this, or elevation 38, while the bottom of the well is at elevation 8. During times of heavy pumping the water level is drawn down to about elevation 20. At the time of the recent investigation the ground water immediately surrounding the well was at about elevation 53, while at a point about 50 feet westerly of the well ground water was not encountered at elevation 40.

*"Pumping machinery.*—The water from this well is pumped by two electrically operated pumps in the pump pit located about 50 feet southeast of the well. This pump pit is 25 feet in diameter and about 35 feet deep. Each of these pumps has a capacity of 700,000 gallons per day and they are so arranged that either one can be used for ordinary day service and both in series when it is desired to raise the pressure for fire service.

*"Sewers and surroundings.*—There is an eight-inch house sewer which runs from N Street through the alley west of 24th Street and across private property back of the pump pit to a manhole south of N Street. From there it runs directly past the wall inclosing the entrance to the boiler house and across N Street to the manhole in the alley between N and O Streets. Besides this sewer a number of outhouses are in close proximity to the well and there are also other features, such as filling-in of the surrounding ground with street cleanings and other rubbish and the depression caused by changing the course of the Antelope Creek, which make the surroundings unsightly and unsanitary. There are also numerous pipes used for drains, blow-offs, and other purposes at the time the plant was steam-operated, leading out from the well and for the most part forgotten at present, which would form easy routes by which the ground water might enter the well.

*"Quality of the water.*—We have no chemical analysis of this water, but from bacterial analyses made from time to time by Dr. Waite and others it is apparent that this water is not ordinarily contaminated by seepage of water into the well. From the fact that ground water about the well, however, is drawn down to a considerable depth and that there is no outlet for the considerable amount of surface wash coming into the hollow back of the station and into the old water course of the Antelope Creek, we should expect that during times of heavy rain a considerable amount of foreign matter could be and undoubtedly is carried into the well."

#### INVESTIGATION OF FIRST EPIDEMIC.

During the first three weeks of this investigation samples of water were collected and examined from all three of the city wells without finding any evidence of contamination or infection. The localization of the outbreak and the surroundings of the Rice well were constantly kept in mind, since a contamination of this well would have furnished the most probable source of infection.

Repeated examinations, however, failed to give any evidence whatever of contamination. This was true not only when the water was collected at the pump but also when it was collected from the well itself. At this time there was very little seepage and at no place was the amount sufficient for collection from the wall itself. In order to get a sample it was necessary to scrape the wall. This was done in several places where the wall was covered with a brown, moist layer which appeared to be composed chiefly of iron rust. In these scrapings there was absolutely nothing to indicate pollution.

Having found nothing in the water from the wells, an examination was made of the water from the faucets in different parts of the city but especially in that part where the majority of the cases existed. Here evidence of contamination in the mains was soon found. During the last two weeks in September samples of water were obtained from 14 of the city schools. No colon bacilli were found in the samples from 11 of the schools. Colon bacilli were present in one cubic centimeter amounts in every one of 10 samples taken from two schools and in one cubic centimeter amounts in five of 10 samples taken from one school. The three schools where colon bacilli were found were all in the section where the epidemic was most prevalent. Since colon bacilli were not found in water from the schools outside the area where the disease prevailed nor from other places outside this area, at this time, numerous samples were collected at various places covering the entire section involved. Colon bacilli were found in one cubic centimeter amounts in more than 50 per cent of the samples collected. Since there was evidence of contamination of the water in the mains in the area presumably supplied by the Rice well, an attempt was made to find out where this contamination came from. At this time there were two possible explanations for the source of contamination: one that the mains were being or had been contaminated directly, the other that they had been contaminated by the Rice well some time before the investigation was begun. It has been shown conclusively in several instances that direct contamination of mains may occur under certain conditions. No positive evidence of conditions favorable to such contamination was obtained during this

investigation. In the light of what was found at the time the second epidemic was investigated the evidence is convincing that the most probable explanation of the contamination of the city mains in northeast Lincoln during the time when such contamination was found to exist was through the Rice well.

That evidence of contamination of the Rice well at this time was not found is easy of explanation. The investigation was not begun until August 30. A very large proportion of those having the disease were attacked about the middle of August. The contamination which caused this infection began at least from one to three or more weeks before this time. There was practically no precipitation in July except on July 9 and 23. At this time the condition of the soil was such that seepage of surface water might have occurred very easily. The total precipitation for August 2, 3, and 4 was 1.16 inches. A contamination of the water at this time, if it occurred—and it is quite probable that it did—would have taken place at just about the right time to explain the large number of those attacked about August 20, since it would correspond quite closely with the usual typhoid incubation period. Since the investigation was not begun until the last of August, there was abundant time for all evidence of contamination to have disappeared from the well, and it would not have been at all unusual for it to have disappeared from the mains.

Following the request of the Governor, Dr. L. L. Lumsden, passed assistant surgeon of the United States Public Health and Marine Hospital Service, was detailed by the Surgeon General to proceed to Lincoln, Neb., to make an investigation of an outbreak of typhoid fever in the city and to determine the sources of infection and the measures necessary for the control of the disease.

Dr. Lumsden began his investigation on November 4 and terminated it on November 11, 1911. During this investigation the colon bacillus was encountered for the first time in water taken from the A Street well on November 10 in 10 c.c. amounts of water. It was found a number of times after this during Dr. Lumsden's investigation and since then I have obtained it repeatedly in samples from this well up to the first of August, 1912.

<sup>1</sup> *Public Health Report*, 1912, 27, No. 21.



From November 18, 1911, to December 5, 1912, 57 samples of water were secured from the A Street well and its surroundings. Three of these were taken from pools in the more or less immediate vicinity of the well. A sample was taken from standing water which collected chiefly from the overflow of a watering trough situated at a distance of from 125-50 feet east of the well. This water disappeared although it had no visible outlet. Colon bacilli were present in this water in large numbers. A second sample was taken from the Antelope at a point where some of the waste water from the pumping-station empties into the creek. This sample was taken at a distance of about 100 yards east of the well. There were many colon bacilli present in this sample. A third sample was taken from a pool near the driveway northeast of the well. In this sample, colon bacilli were also present in lesser numbers than in the other two samples. Colon bacilli have never been found in the seepage of the A Street well except in that which comes from the wall on the east. The seepage here is very slight compared with that which comes from the wall on the west and south. Seepage occurs only on the walls of the middle and bottom section of the well. In the many samples of seepage collected from both sections of the well, colon bacilli have been found in only one sample from the middle section. At this time they were present in one cubic centimeter of the seepage. A large percentage of the samples collected from the bottom section show colon contamination. Since colon bacilli are rarely encountered in seepage from the middle section of the well and are of frequent occurrence in samples from the bottom section, it seems logical to suspect that the contamination does not come from surface washings directly, since seepage begins at a distance of about 30 feet below the surface and colon bacilli are not found until a depth of 60 feet or more is reached. A series of experiments had been planned to determine if possible the source of this contamination. Very soon after they were begun they had to be abandoned, since the time necessary to carry out these plans was required in the investigation of the epidemics which began about the middle of December. The results of the examinations of the water from the three pools in

the vicinity of the A Street well and from the seepage in the A Street well are as follows:

RESULTS OF EXAMINATIONS OF WATER FROM POOLS AND FROM SEEPAGE FROM THE A STREET WELL.

SOURCE	DATE OF EXAMINATION	NUMBER OF BACTERIA PER CUBIC CENTIMETER ON AGAR, AFTER 48 HOURS' INCUBATION AT 37.5° C.	GAS IN LACTOSE BROTH FROM		<i>B. coli</i> IN	
			I C.C.	10 C.C.	I C.C.	10 C.C.
Pool nearest A well. . . . .	1911 Nov. 18	1,500	+	+	+	+
Pool northeast of A well. . . . .	" "	155	+	+	+	+
Antelope Creek, east. . . . .	" "	240	+	+	+	+
Seepage, east, M. . . . .	" "	4	-	-	-	-
" northeast, M. . . . .	" "	3	-	-	-	-
" west, M. . . . .	" "	3	-	-	-	-
" southeast, B. . . . .	" 20	1,000	-	+	-	+
" east, B. . . . .	" "	1,200	+	+	+	+
" northeast, B. . . . .	" "	80	+	+	+	+
" west, B. . . . .	" "	10	-	-	-	-
" southwest, B. . . . .	" "	8	-	+	-	+
" southeast, M. . . . .	" "	15	+	+	+	+
" northeast, M. . . . .	" "	7	-	-	-	-
" north, M. . . . .	" 21	7	-	-	-	-
" south, M. . . . .	" "	4	-	-	-	-
" east, M. . . . .	" "	25	-	-	-	-
" east, M. . . . .	" "	1	-	-	-	-
" east, 1, B. . . . .	" 22	2,000	+	+	+	+
" east, 2, B. . . . .	" "	220	+	+	+	+
" east, 3, B. . . . .	" "	110	+	+	+	+
" east, 4, B. . . . .	" "	4	-	-	-	-
" southeast, B. . . . .	" "	8	-	-	-	-
" northeast, B. . . . .	" "	10	-	-	-	-
" east, 1, B. . . . .	" 23	1,185	+	+	+	+
" east, 2, B. . . . .	" "	190	+	+	+	+
" east, 3, B. . . . .	" "	575	+	+	+	+
" east, 4, B. . . . .	" "	305	+	+	+	+
" east, 5, B. . . . .	" "	365	-	+	-	-
" east, 1, M. . . . .	" "	4	-	-	-	-
" east, 2, M. . . . .	" "	15	-	-	-	-
" east, 3, M. . . . .	" "	5	-	-	-	-
" east, 1, B. . . . .	" 24	540	+	+	+	+
" east, 2, B. . . . .	" "	1,260	+	+	-	+
" east, 3, B. . . . .	" "	435	+	+	+	+
" east, 4, B. . . . .	" "	135	-	-	-	-
" east, 1, M. . . . .	" "	8	-	-	-	-
" east, 2, M. . . . .	" "	38	-	-	-	-
" east, 3, M. . . . .	" "	30	-	-	-	-
" east, 1, B. . . . .	" 27	450	+	+	-	+
" east, 2, B. . . . .	" "	500	+	+	-	+
" east, 3, B. . . . .	" "	370	+	+	+	+
" east, 1, B. . . . .	" 28	140	+	+	+	+
" east, 2, B. . . . .	" "	55	+	+	-	+
" east, 3, B. . . . .	" "	250	+	+	-	-
" east, 4, B. . . . .	" "	100	-	+	-	+
" east, 1, M. . . . .	" "	10	-	-	-	-
" east, 2, M. . . . .	" "	25	+	+	-	-
" east, 3, M. . . . .	" "	35	-	-	-	-
" east, 1, B. . . . .	1912 Dec. 2	1,500	-	+	-	+
" east, 2, B. . . . .	" "	100	+	+	-	-
" east, 3, B. . . . .	" "	1,250	+	+	+	+
" east, 4, B. . . . .	" "	325	+	+	-	+
" east, 1, M. . . . .	" 5	25	-	-	-	-
" east, 1, B. . . . .	" "	80	+	+	-	-
" east, 2, B. . . . .	" "	75	-	+	-	-
" east, 3, B. . . . .	" "	35	-	+	-	-
" east, 4, B. . . . .	" "	150	-	+	-	-
Average. . . . .		301	..	..	..	..
Percentage, positive. . . . .		.....	56	68	30	50

## INVESTIGATION OF SECOND EPIDEMIC.

On the 14th of December, 1911, a sudden and explosive outbreak of a more or less severe intestinal disease took place in Lincoln. Opinions as to what it was and from whence it had arisen were many. The following names were given to it: "winter grip," "influenza," "ptomaine poisoning," and "winter cholera." A number asserted with no uncertain positiveness that it was caused by a change in weather conditions and was transmitted through the air. The city physician vehemently declared that it was due to the eating of "tainted veal." Since the individuals affected were even more closely confined to the area presumably supplied with water from the Rice well than in the first epidemic of typhoid fever, some asserted, including myself, that it was most probably due to a contaminated water supply in the area involved. The 14th of December was chosen arbitrarily as the date of the beginning of the outbreak of "winter cholera," since the number affected on this date seems to have been greater than upon any other date and few, if any, were affected before this time. That influenza or a similar infection was also epidemic at this time is beyond question. This latter infection, however, certainly began before December 14 and was quite generally distributed over the entire city. No history of diarrhea was obtained in any of those who were afflicted with "influenza."

On the 18th of December the City Council of the city of Lincoln requested me to investigate this outbreak and determine its cause if possible. A canvass of about 50 individuals who were afflicted or had been afflicted with this intestinal trouble revealed the fact that more than 90 per cent had not partaken of veal within a week or more before the outbreak. This would eliminate veal from consideration in the majority of those who had the disease.

*Description of the disease.*—The number of individuals living in the area where the outbreak occurred is approximately 15,000. Of this number it would be conservative to estimate that 3,000 or more were affected to a greater or less degree. While collecting water from 11 houses in the district involved, three families numbering collectively 27 were all affected and nearly all of these dated the beginning of their trouble on December 14. In nearly all the

other houses one or more were affected. In making a detailed investigation of 50 cases of typhoid fever which subsequently developed, data were obtained concerning the outbreak of "winter cholera." Thirty-three of the typhoid patients had suffered an antecedent attack of "winter cholera," making for the number investigated a total of 66 per cent.

In the majority of those affected no temperature occurred or was so slight that it was not observed. In two it was said to have reached  $104^{\circ}$ . One had a maximum temperature of  $102^{\circ}$ , 37 had a slight temperature, 67 had none at all. The duration of the trouble was one or two days in the majority of cases, in some it lasted for two weeks. A duration of more than five days was infrequent. The attack was sudden in most instances. The pains varied in intensity from slight to severe. They were described as occurring in periodic, griping paroxysms. The stools were frequent, watery, and contained mucus. In a few cases blood was present in the evacuations. In one instance there was prolapse of the rectum.

The date of attack in 107 cases was as follows:

1911		1911	
December 14.....	34	December 21.....	5
" 15.....	24	" 22.....	4
" 16.....	10	" 24.....	2
" 17.....	1	" 25.....	6
" 18.....	3	" 29.....	3
" 19.....	1	" 31.....	4
" 20.....	10		
		Total.....	107

The numbers given above were practically definite for the 14th, 15th, and 16th of December. The numbers and dates for the other cases were not so definitely determined. Death occurred only in those at the extremes of life. The ages and sex of those who died were as follows:

AGE	SEX	
	Male	Female
83 years.....	..	1
78 ".....	..	1
73 ".....	..	1
72 ".....	..	1
1 year.....	1	..
7 months.....	1	..
3 ".....	1	..
Total.....	3	4

During the four months July, August, September, and October, 1911, 161 cases of typhoid fever were reported in the city of Lincoln. Of this number 141 were investigated and 144 were considered in detail. The results of this investigation appear in Dr. Lumsden's report of the first epidemic.

In November, 1911, the following cases of typhoid were reported in Lincoln:

November 7.....	1 case	November 21.....	1 case
" 13.....	1 "	" 26.....	1 "
" 17.....	1 "		
Total..... 5 cases			

In December the first reported case was on the 15th. This case should not be included in the second epidemic, its onset having occurred before the cause of this outbreak had originated.

The individuals affected were according to sex and age as follows:

AGE AND SEX OF PERSONS AFFECTED IN FIVE-YEAR PERIODS, IN LONGER PERIODS, AND OF THOSE WHO DIED.

Numbers Affected in Five-Year Periods	Male	Female	Total	Age in Years	Percentage	
0 to 4.....	8	12	20	0 to 14.....	30.7	
5 to 9.....	20	32	52	10 to 20.....	67.3	
10 to 14.....	33	26	59	0 to 20.....	89.1	
15 to 19.....	38	34	72	30 to 69.....	10.9	
20 to 24.....	32	32	64	DEATHS		
25 to 29.....	16	11	27	Age	Male	Female
30 to 34.....	9	9	18	5.....	1	1
35 to 39.....	2	6	8	11.....	1	0
40 to 44.....	3	1	4	14.....	0	1
45 to 49.....	1	1	2	15.....	2	0
50 to 54.....	1	1	2	16.....	0	1
55 to 59.....	0	0	0	18.....	2	0
60 to 64.....	0	1	1	20.....	1	1
65 to 69.....	0	1	1	24.....	0	1
Totals.....	163	167	330	28.....	0	1
				30.....	1	0
				43.....	1	0
				53.....	1	0
				Total.....	10	6
				Mortality.....	4.85 per cent	

GEOGRAPHICAL DISTRIBUTION IN THE TWO EPIDEMICS OF TYPHOID FEVER.

Of all the cases reported and investigated which occurred during July, August, September, and October, 1911, about 84 per cent were in the district north of J Street and east of 14th Street. Of the

cases reported from July 1 to December 1, 1911, 76.5 per cent occurred in the area north of Randolph Street and east of 14th Street. Of the cases reported in the second epidemic from December 1, 1911, to March 1, 1912, 86 per cent occurred in this same area. The geographical distribution of cases alone would indicate that the cause of the trouble was definitely localized. Since the second epidemic occurred in the winter months, contact, flies, etc., played a lesser rôle than they did in the first epidemic and hence we have a larger percentage confined to the area bounded on the south by Randolph Street and on the west by 14th Street.

#### SOURCE OF INFECTION.

On the 18th of December, 1911, samples of water were collected from 1619 R Street, 1645 R Street, 2109 Q Street, 2625 P Street, and from the Rice well which is located between 23d and 24th Streets near N Street. All of these samples contained colon bacilli in 10 cubic centimeter amounts and that from 1619 R Street in one cubic centimeter. Never before in the numerous examinations of samples of water from the Rice well had the colon bacillus been encountered. A search was made to find out if possible where the contamination came from and it was discovered that water was being discharged into the well from a six-inch pipe situated a little north of west in the wall of the Rice well about 15 feet below the surface. Samples taken directly from this pipe showed gross pollution. No information could be obtained as to where this pipe came from and for what purpose it entered the well.

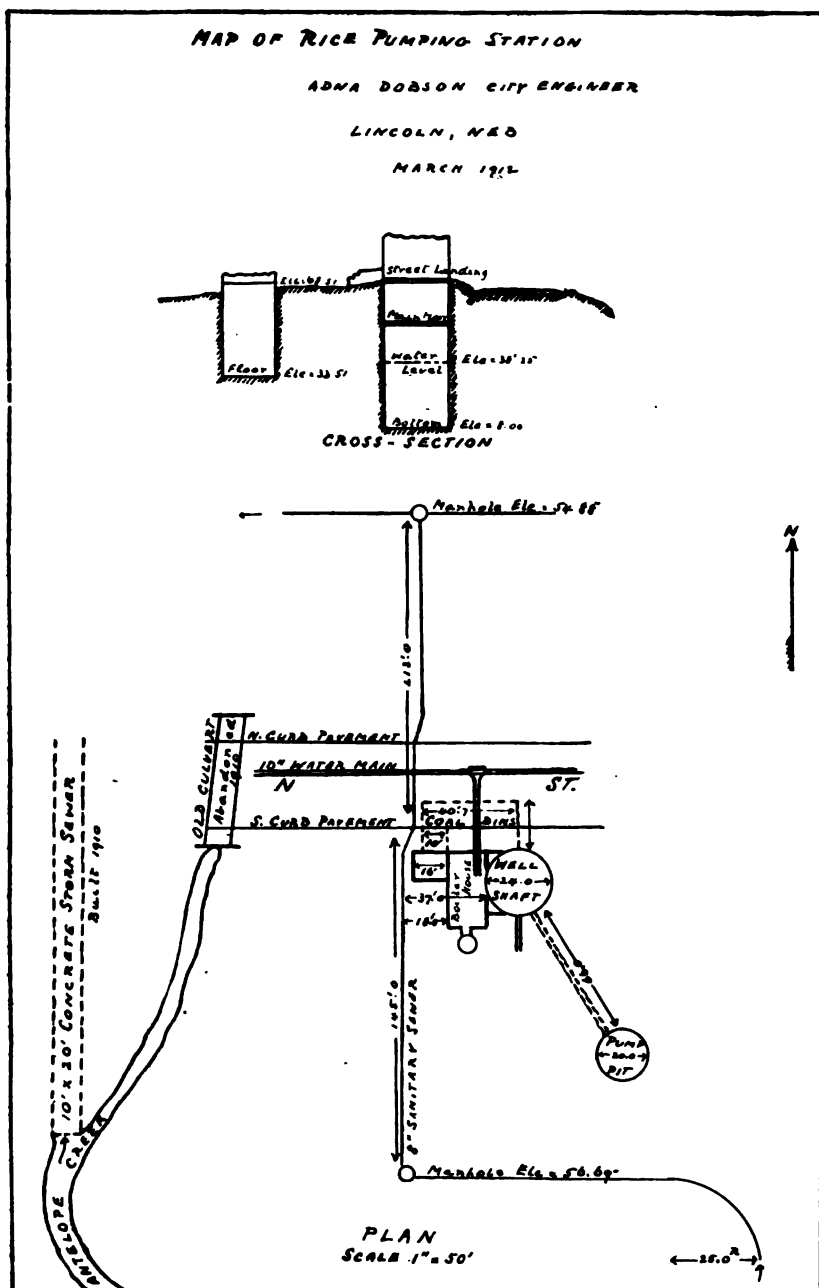
On December 20, it was found that this pipe was certainly the main source of contamination of the Rice well, and the latter was permanently abandoned as a source of water supply. A large part of the wall of the well was moist, the moisture being due to seepage; though at no place was there enough trickling to obtain a sample of the seepage water.

On December 21, excavation was begun for the purpose of ascertaining the whereabouts of the pipe outside the well through which polluted seepage was entering. It was found to end about 14 or 15 feet outside the well. The end outside the well was open. Earth had entered this open end for a few inches, otherwise the

inside of the pipe was clear. Samples of water taken from the seepage which collected at the open end as well as along the pipe showed evidence of gross pollution. Notwithstanding the fact that the water level toward the west was apparently lower than the open end of the pipe outside the well, suspicion was aroused that the seepage water which collected around the open end of the pipe outside the well might come from the sewer which ran from south to north on the west side of the well. This sewer pipe was at a distance of only 37 feet from the wall of the well and consequently only 22 or 23 feet from the open end of the pipe which entered the well. Besides this sewer there were other things in the immediate surroundings of this well of a very objectionable character. At a relatively short distance from this well to the south and east there is a low spot which the city has been filling with street sweepings for a considerable period of time. It has also served as a dumping-ground for other refuse. In the immediate vicinity of this dumping-ground there are still a number of open privies in use at the present time. Rain water or water from any other source not only might but probably would carry material from this dumping-ground and from these privies into the well.

The combined rainfall of December 9 and 10 was 1.40 inches. It is highly probable that this precipitation was the chief cause which brought about the contamination of the Rice well. At no time when the investigation of the water was being made from August 29 to December 18 was anything of a suspicious character found in the Rice well. Why contamination did not occur after some of the heavy rainfalls during August, September, October, and November is difficult to explain.

At the time the first sample of water was taken from the pipe which was contaminating the Rice well it was estimated that the amount of seepage which was entering the well through this pipe was one quart per hour. This estimated amount was determined from the amount which was actually collected and measured after an interval of 10 minutes, on December 19. The amount was certainly greater on the 19th than it was on any subsequent date and it was probably much greater in amount on the days immediately following the rainfall of December 9 and 10.





Early in the investigation of the first epidemic the treatment of the water in the mains by the hypochlorite method was advised, since colon bacilli were continuously present in samples of water collected from faucets. This advice was not accepted at this time. Instead of this, after considerable discussion, the Council directed the water commissioner to open up the water hydrants for the purpose of flushing out the mains. This was done about December 21. The number of colon bacilli in the water mains began to diminish after the closure of the Rice well. It is probable that the flushing contributed to some extent in the lessening of the numbers of colon bacilli in the mains but the factor which contributed to the greatest extent was certainly the abandonment of the Rice well.

The following is from the report of Mr. George W. Fuller, the data for which were obtained by Mr. James C. Harding:

*"Sterilization of A well.*—In the latter part of December Mr. H. A. Whittaker, of Minneapolis, arrived at Lincoln at the request of Mayor Armstrong and installed temporary devices for the introduction of hypochlorite of lime into the water of the A well.

*"Pollution of Rice well.*—As stated, there is an eight-inch sewer leading directly through the water works grounds and within about 30 feet of this well. Examinations of the manholes of this sewer line indicated that at some previous time sewage had risen in the manhole in the alley between N and O Streets to a point some two feet above the top of the sewer. The records in the sewer office were looked over and it was found that a stoppage had occurred on this sewer below this manhole on December 12 and 13. With the assistance of the engineering and sewer departments this sewer was blocked at the manhole and the sewage allowed to rise until it reached the ring of grease and other matters left there by the earlier stoppage of December 12 and 13. An excavation was made inside the areaway leading to the boiler house.

"A short time after the sewage had reached the level above indicated it was seen to enter this excavation from the side next to the sewer. Later two joints of this sewer were uncovered. It was found that they leaked in both cases, giving an opportunity for sewage to escape into the surrounding earth. To the south of the excavation the pipe had apparently been laid on firm material, but to the north of this point it was laid on filling composed mostly of cinders and ashes. There was apparently a slight settlement at one of these joints, and here the earth had been washed away, showing that at some time considerable sewage had leaked out from the pipe at this point.

"Whether or not there are other joints which are in the same condition, we do not know, but it is quite likely that this may be the case. It is also possible that sewage may find its way out of the sewer pipe at the offset made in the sewer just north

\* Mr. Whittaker arrived in Lincoln at 5:45 P.M., January 15, 1912. The administration of hypochlorite was begun on January 17, 1912. The temporary device which was installed at that time was in no way essentially different from the device which has been in continuous operation since it was installed in January up to the present date, September 1, 1912.

of the wall of the coal chamber. In any event it was apparent that sewage could leak from faulty sewer joints within two feet of the wall of the boiler room areaway.

"Excavations were then made to determine if possible by what route this sewage found its way into the well. It was found that just east of the areaway wall there had been in the past a hydraulic hoist for raising the ashes out of the coal chamber. At the time the sewer was blocked on January 24, 1912, this chamber was found to be filled with sewage. From this chamber there was a drainpipe leading into a small manhole near the corner of the boilers. At the time of the investigation the end of this pipe was plugged in the hoist chamber. Whether or not this was the case during August and December, we do not know. But regardless of this there was a reasonably direct passage for the sewage either through this pipe or through the trench in which the pipe was laid along the south wall of the areaway. From the manhole spoken of above there were numerous ways in which the sewage could find ready entrance to the well. There was an abandoned 10-inch drainpipe from this manhole which formerly took the waste water from some sinks located in the boiler house. Directly below this pipe was a six-inch pipe open at both ends and leading directly into the well.

"There was also apparently a free passage beneath the boilers and along the boiler wall to a chamber beneath the end of the east boiler and one side of which was formed by the masonry wall of the well. From this chamber there were four small pipes which led directly into the well. The masonry around the discharge pipe which passes into this chamber was also in poor condition and would also easily admit the passage of sewage. There were also other pipes, used at the time the plant was steam-operated, which have been buried in the ground and long since forgotten.

"That there were many opportunities for the sewage to reach the well was readily apparent, and it was not thought necessary to go further into the matter. Later, if desired, after the frost is out of the ground, it might be possible to block the sewer in the same manner as previously done, and the course of the sewage could perhaps be determined in precise detail.

#### CONCLUSIONS AS TO DECEMBER EPIDEMIC.

"The epidemic of typhoid fever during December, 1911, and January, 1912, can be definitely attributed to the following rather remarkable combination of circumstances, the absence of any one of which might have prevented the trouble:

"On December 9 and 10 there was a rainfall of 1.40 inches, the first of any importance since October 12, when there was a rainfall of 0.92 inch. On December 12 and 13 the sewer passing the well was working under a head, owing to a stoppage in the sewer below. This sewer has at least two faulty joints where the pipe adjoins the west wall of the areaway. The sewage leaking from these joints found its way through the ash hoist chamber and from there through pipes or other routes to the well.

"*August pollution.*—From the appearance of the soil surrounding the sewer joints examined, it was evident that there had been leakage of the sewage in small quantities for some time. The joints were unusually well made, but their condition would indicate that owing to the extreme cold weather at the time this sewer was laid (December, 1910) some of the mortar in the bottom of the joints fell out before the cement had thoroughly set.

"On August 2, 3, and 4, 1911, there were heavy rainfalls which filled the hollow just south of the well to a point somewhat higher than the manhole located at the angle in the sewer line at this point. There is no outlet to this hollow, except as the

water may follow the sewer trench previously referred to or by percolating through the ground. Probably the water during these rain storms followed both courses. Whether the pipe leaked before this time or whether the water following along the trench caused the settlement of the sewer at this time, we do not know.

*"Infection of sewage.*—It was not only necessary to determine that the sewage found its way into the Rice well, but it was also necessary to prove beyond reasonable doubt that there were typhoid bacilli in the sewage. As to the last epidemic, there could be no question but that this condition existed, as there were at least nine authentic cases reported on these sewer lines. For the first epidemic, however, the Health Department had no records as to typhoid in this district, as it has only been during the last six months that the physicians have been compelled by the Health Department to report all cases of typhoid of which they had knowledge. On the request of Mr. Harding the Health Department made a house-to-house canvass of the entire district which drained into the sewer system leading past the well. The result of this canvass proved conclusively that there were typhoid patients in houses connected with this sewerage system."

#### RESULTS OF THE EXAMINATIONS OF THE CITY WATER FROM DECEMBER 18 TO DECEMBER 28, 1911.

It had been intended to incorporate in this record the result of all the examinations of city water made from December 18, 1911, up to and including the time when hypochlorite was first introduced into the water mains. Through accident the record for the first half of the month of January, 1912, was lost. In general it agreed with that for the latter part of December, 1911, the only difference being that the degree of contamination became continuously less. (See table on p. 33.)

#### HYPOCHLORITE TREATMENT OF THE CITY WATER SUPPLY.

On the 15th of January, 1912, Mr. H. A. Whittaker, chemist and bacteriologist of the Minnesota State Board of Health, arrived in Lincoln in response to a request received from A. H. F. Armstrong, mayor of the city of Lincoln, to treat the water with the hypochlorite method.

The quotation is from Mr. Whittaker's report to the mayor and City Council of the city of Lincoln.

#### INSTALLATION OF THE EMERGENCY PLANT.

"The place selected for installing the plant and administering the hypochlorite was the A Street pumping-station, since a large proportion of the water was distributed from this source. The F Street station at this particular time was used only intermittently and furnished a small part of the city supply. It was therefore assumed that during the

RESULTS OF THE EXAMINATIONS OF THE CITY WATER FROM DECEMBER 18 TO  
DECEMBER 28, 1911.

SOURCE	DATE OF EXAMINA- TION	NUMBER OF BACTERIA PER CUBIC CENTI- METER ON AGAR, AFTER 48 HOURS' INCUBATION AT 37.5° C.	GAS IN LACTOSE BROTH FROM		<i>B. coli</i> IN	
			1 c.c.	10 c.c.	1 c.c.	10 c.c.
2625 P Street.....	Dec. 18	2	—	+	—	+
2109 Q ".....	" "	4	—	+	—	+
1619 R ".....	" "	10	+	+	+	+
1645 R ".....	" "	3	—	+	—	+
Rice well.....	" "	20	—	+	—	+
2144 B Street.....	" 19	4	—	—	—	—
A Street well.....	" "	15	—	—	—	—
East faucet A Street well.....	" "	4	—	+	—	—
F Street well.....	" "	3	—	—	—	—
Drip from lower platform in Rice well.....	" "	{ Colon 3,550	+	+	+	+
Pump at Rice well.....	" "	{ Colon 300	—	—	—	—
Exhaust pipe in Rice well.....	" "	{ Colon 4	—	—	—	—
Laboratory faucet.....	" "	{ Colon 17	+	+	+	+
1811 L Street.....	" 20	{ Colon 130	—	+	—	—
Rice well.....	" "	{ Colon 4	—	—	—	—
Exhaust pipe in Rice well.....	" "	{ Colon 20	+	+	+	+
Seepage in soil outside Rice well. . .	" 21	{ Colon 1	+	+	+	+
Seepage inside near old engines of Rice well.....	" "	{ Colon 100	+	+	+	+
Seepage in coal room.....	" "	{ Colon* 1,000	+	+	+	+
Exhaust pipe in Rice well.....	" "	{ Colon* 375	+	+	+	+
216 North 26th Street.....	" "	{ No plates	—	—	—	—
2715 R Street.....	" "	{ Colon 10	+	+	+	+
2727 P ".....	" "	{ Colon 6	—	+	—	+
2701 P ".....	" "	{ Colon 1	—	+	—	+
110 South 28th Street.....	" "	{ Colon 2	—	+	—	+
3090 R Street.....	" "	{ Colon 5	—	+	—	+
Outer end of pipe outside Rice well..	" "	{ Colon 4	—	+	—	+
430 North 26th Street.....	" 27	{ Colon* 3,550	+	+	+	+
558 ".....	" "	{ Colon 10	+	+	+	+
2509 Vine Street.....	" "	{ Colon 2	—	+	—	+
2346 ".....	" "	{ Colon 0	—	+	—	+
2212 ".....	" "	{ Colon 0	—	+	—	+
320 North 26th Street.....	" "	{ Colon 0	—	+	—	+
337 South ".....	" 28	{ Colon 1	+	+	+	+
403 ".....	" "	{ Colon 4	—	+	—	+
235 ".....	" "	{ Colon 6	—	+	—	+
2332 N Street.....	" "	{ Colon 2	—	+	—	+
205 South 25th Street.....	" "	{ Colon 2	—	+	—	+

\* Too numerous to count in 1 c.c. of water. Colon colonies counted from cultures grown on separate lactose, litmus agar plates. Colonies producing both gas and acid on these plates were considered colonies of the *B. coli*.

periods the F Street station was not in operation the treated water from the A Street station would reach a large portion at least of the section supplied from the F Street station. Furthermore, bacteriological examinations showed the absence of contamination at the two sources and its presence only in the distribution system on dates just preceding the date of installation of this plant.

"With the assistance furnished by the officials through the various municipal departments, the emergency plant was installed and began to administer chemical at 3:00 P.M., January 17.

"In the emergency disinfection of public water supplies it is advisable to add more hypochlorite or chloride of lime than would ordinarily be used for a continuous treatment of the same water. This excess amount prolongs the disinfecting power of the chemical in solution, thereby destroying the vegetative forms of bacteria in parts of the system remote from the point of addition. Such addition, although harmless, often causes odor and taste in the water, such as was true in Lincoln, especially during the first few days of treatment. In confining the treatment to the destruction of organisms at the point of addition, much smaller quantities can be used which should not give rise to a disagreeable odor and taste. This point is mentioned here to make clear the fact that strong odor and taste is not usually necessary where a continuous treatment is applied.

"Table 1 gives a record of the amount of chemical added to the water each day the work was under my supervision. The term 'available chlorine' is used in the table to designate the active part of the chemical or that present in the form of hypochlorite. These figures represent the amounts as closely as could be determined under existing conditions. As indicated by the table, the amount of chemical applied was reduced considerably during the period of the treatment, as it was found that good results could be secured with less hypochlorite. On January 23 the amount of chemical was reduced to one-half of the amount indicated on the last date mentioned in the table. As the previous treatment had shown such excellent results and the bacteriological examinations were to be continued on the water, it was considered safe to reduce the amount to this point and thus eliminate the odor and taste as much as possible.

TABLE 1.  
AMOUNT OF CHEMICAL ADDED TO THE WATER.

DATE	GALLONS OF WATER PUMPED	POUNDS PER MILLION GALLONS		AMOUNT OF AVAILABLE CHLORINE ADDED	
		Chemical	Available Chlorine	Grains per Gallon	Parts per Million
3:00 P.M. to 3:00 P.M.					
Jan. 17 to Jan. 18.....	3,312,925	25.9	7.4	0.049	0.84
" 18 " " 19.....	3,270,556	20.6	5.9	.030	.68
" 19 " " 20.....	3,313,676	16.0	4.5	.030	.52
" 20 " " 21.....	3,066,184	17.2	4.9	.032	.56
" 21 " " 22.....	3,124,520	16.4	4.7	.031	.54

#### BACTERIOLOGICAL CONDITION OF WATER BEFORE AND AFTER TREATMENT.

"In order to understand clearly the bacteriological results, it will be necessary to outline briefly the methods used in carrying out this work. The samples of water examined were collected from flamed taps, either in bottles or flasks especially sterilized for that purpose. The medium used was made in accordance with the standards prescribed by the Laboratory Section of the American Public Health Association for the bacteriological examination of water. The bacterial counts were made on plates incubated at room temperature or at 37.5° C. for 48 hours. The presumptive test for *B. coli* consisted in inoculating the stated amount of water into lactose broth, the total absence of gas formation at the end of 48 hours' incubation at 37.5° C. being considered a negative test for *B. coli*. Such tubes as showed gas were plated on litmus, lactose agar, and incubated for 24 hours at 37.5° C., the absence

of red colonies being also considered a negative test for *B. coli*. These samples stated as positive for *B. coli* showed gas formation and also gave red colonies on litmus, lactose agar. As from 10 to 14 days is required to isolate and identify the colon organisms, it was impossible to make complete examinations on these samples giving positive presumptive tests.

"Before taking up the discussion of the results, I wish to state that a large share of the bacteriological work was done in co-operation with Dr. H. H. Waite, who at all times rendered me valuable assistance.

"In order to determine analytically whether or not indications of contamination were present in the system, samples were collected from various parts of the city before chemical treatment of water was commenced. These results are tabulated in detail in Table 2 attached to this report.

"Samples 1 to 12 inclusive were collected on January 17 before the chemical was added or had time to affect the water in the system. As indicated by these results, presumptive tests for *B. coli* were obtained on samples 2 and 5 in 1 c.c. amounts and on samples 1, 3, 4, 6, 9, 10, 11, and 12 in 10 c.c. amounts.

"The bacterial counts on the samples just mentioned are relatively low, yet the indication of *B. coli* in this low count points analytically to the bacteria present being largely of fecal origin. An opinion based on the presumptive test for *B. coli* shows the presence of contamination at the points mentioned in the system before treatment began. Attention should be called to sample 7 from the A street well and sample 8 from the F Street well, which show the absence of *B. coli* in 100 c.c. amounts and the total absence of bacteria in 1 c.c. plates made for the bacterial count. This fact is significant, as it indicates that on the date these samples were collected the infection appeared to be in the distribution system, but not in the wells.

"Samples 13 to 48 inclusive, collected after the chemical had been added to the water, show the total absence of bacteria in 1 c.c. amounts in most of the samples collected, and the presumptive test for *B. coli* did not indicate the presence of this organism in 100 c.c. amounts. These samples, as Table 2 indicates, were distributed quite thoroughly over the entire distribution system, and an attempt was made to collect samples from dead ends after flushing to determine if the infection had been removed from these remote parts of the system.

"It is interesting to note that the contamination on January 17, in samples 1, 2, 3, 4, 5, 6, 9, 10, 11, and 12, had entirely disappeared on January 18 about 24 hours after treatment had begun, as shown by samples 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, and 24, collected from the same points on the system. These results would justify the opinion that the distributing system had been freed from all evidences of contamination at these points. The results obtained from the examination of 24 additional samples, 25 to 48 inclusive, from other points scattered widely over the system, justified this assumption.

#### CONCLUSION OF RESULTS ON TREATMENT OF THE WATER.

"It was decided, on the basis of the excellent bacteriological results secured by treatment of the city water by hypochlorite, that it was unnecessary for me to remain in Lincoln. The permanent safety of the wells seemed to be questionable as judged from local data, and it appeared best to leave the emergency plant in operation until such a time as the city could replace this plant or be assured that the city wells could be depended upon to furnish a water of safe sanitary quality.

"On January 22 I appeared before the City Council and explained the work which had been done during the week and stated that the disinfection work on the system was practically completed.

TABLE 2.  
BACTERIOLOGICAL CONDITION OF WATER BEFORE AND AFTER TREATMENT.

NUMBER	COLLECTED		SOURCE	BACTERIA PER C.C.		PRESUMPTIVE TEST FOR <i>B. coli</i>		
	Date	Hour		R.T.*	37.5°C.	1 c.c.	10 c.c.	100 c.c.
1.....	1-17-12	11:30 A.M.	635 S. 29th Street	..	0	0	+	+
2.....	"	11:45 A.M.	149 S. 29th "	..	0	0	+	+
3.....	"	11:55 A.M.	2839 R. "	..	7	0	+	+
4.....	"	12:10 P.M.	2501 R. "	..	45	0	+	+
5.....	"	12:20 P.M.	2110 R. "	..	8	+	+	+
6.....	"	1:30 P.M.	2144 B. "	..	0	0	+	+
7.....	"	2:00 P.M.	A Street well	..	0	0	0	0
8.....	"	3:00 P.M.	F Street well	..	0	0	0	0
9.....	"	3:10 P.M.	843 S. 10th Street	..	2	0	+	+
10.....	"	3:30 P.M.	1408 F. "	..	2	0	+	+
11.....	"	3:40 P.M.	1537 F. "	..	2	0	+	+
12.....	"	4:30 P.M.	Laboratory faucet	..	0	0	+	+
13.....	1-18-12	11:30 A.M.	709 S. 29th Street	0	..	0	0	0
14.....	"	11:35 A.M.	635 S. 29th "	0	..	0	0	0
15.....	"	11:45 A.M.	149 S. 29th "	0	..	0	0	0
16.....	"	12:10 P.M.	2839 R. "	0	..	0	0	0
17.....	"	12:20 P.M.	2501 R. "	0	..	0	0	0
18.....	"	12:30 P.M.	2110 R. "	0	..	0	0	0
19.....	"	1:30 P.M.	2144 B. "	0	..	0	0	0
20.....	"	2:00 P.M.	A Street well	0	..	0	0	0
21.....	"	2:30 P.M.	1537 F Street	0	..	0	0	0
22.....	"	2:45 P.M.	1408 F. "	0	..	0	0	0
23.....	"	3:00 P.M.	F Street well	1	..	0	0	0
24.....	"	3:15 P.M.	Laboratory faucet	0	..	0	0	0
25.....	1-19-12	4:30 P.M.	11th and A Streets	12	4	0	..	0
26.....	"	4:35 P.M.	17th and A "	0	0	0	..	0
27.....	"	4:50 P.M.	29th and Randolph "	0	0	0	..	0
28.....	"	4:50 P.M.	14th and G "	0	0	0	..	0
29.....	"	5:00 P.M.	35th and P. "	0	0	0	..	0
30.....	"	5:15 P.M.	27th and R. "	0	0	0	..	0
31.....	"	5:25 P.M.	35th and T. "	0	0	0	..	0
32.....	"	5:40 P.M.	30th and Oak "	0	7	0	..	0
33.....	"	5:45 P.M.	27th and Hitchcock "	2	28	0	..	0
34.....	"	5:50 P.M.	23d and Holdredge "	3	1	0	..	0
35.....	1-20-12	3:15 P.M.	9th and Wood "	0	0	0	..	0
36.....	"	3:45 P.M.	14th and Marion "	0	0	0	..	0
37.....	"	4:10 P.M.	26th and South "	0	0	0	..	0
38.....	"	4:20 P.M.	33d and Vine "	0	0	0	..	0
39.....	"	4:25 P.M.	27th and Holdredge "	0	0	0	..	0
40.....	"	4:35 P.M.	25th and T. "	0	0	0	..	0
41.....	"	4:40 P.M.	445 S. 25th Street	0	0	0	..	0
42.....	"	4:45 P.M.	25th and Q Streets	0	0	0	..	0
43.....	"	5:00 P.M.	25th and O "	0	0	0	..	0
44.....	"	5:05 P.M.	25th and N "	0	0	0	..	0
45.....	"	5:10 P.M.	25th and M "	0	0	0	..	0
46.....	"	5:15 P.M.	22d and Vine "	0	0	0	..	0
47.....	1-21-12	5:35 P.M.	30th and Oak "	0	0	0	..	0
48.....	"	5:45 P.M.	1805 N. 30th Street	0	0	0	..	0

\* R.T. indicates room temperature.

The (+) sign indicates a positive and the (o) sign a negative presumptive test for *B. coli*.

"On January 23 arrangements were made for leaving the plant under the supervision of local authorities in immediate charge of men trained for this purpose during my stay in Lincoln.

#### RECOMMENDATIONS.

"1. Since the hypochlorite treatment of the city water of Lincoln has proven so efficient in removing the indicated infection from the system, I recommend that the

treatment be continued as long as there is any doubt concerning the sanitary quality of the water furnished from the various sources of supply.

"2. I recommend that daily bacteriological examinations be made of the city water to check the hypochlorite treatment and that frequent examinations should be continued in case chemical treatment is stopped, in order to immediately indicate subsequent contamination.

"3. I recommend that the amount of chemical added to the water be maintained at a point having a reasonable factor of safety to care for unforeseen contaminations.

"In concluding this report I wish to express my sincere thanks to the mayor and City Council for their support and confidence during the prosecution of this work. I wish to express my appreciation to the heads of the municipal departments for their efficient help and for the courtesy and assistance of their subordinates. I am much indebted to the University of Nebraska for placing at my disposal . . . the laboratories in which the bacteriological work of this report was conducted."

(Signed) H. A. WHITTAKER

During the past six months there have been reported at the health office of the city of Lincoln 11 cases of typhoid fever. There is every reason to believe that a greater number of cases than have been reported has not existed in the city during this interval. Only one death from typhoid fever has been reported. The cause of death in this instance was very probably not typhoid fever.

The following table gives the number of cases reported as typhoid fever, by months, as recorded at the city health office from March 1, 1912, to September 1, 1912:

CASES REPORTED AS TYPHOID FEVER, BY MONTHS, FROM MARCH 1, 1912, TO SEPTEMBER 1, 1912.

March	April	May	June	July	August	Total
2	2	1	1	3	2	11

It is highly probable that this number would have been much greater had not the use of the Rice well been discontinued. The continuous use of hypochlorite may also have had something to do in diminishing the number of reported typhoid fever cases.

In conclusion I wish to thank Mr. John J. Putnam for the assistance which he gave me while making these investigations. I wish also to acknowledge my obligations to Dr. L. L. Lumsden, passed assistant surgeon, Public Health and Marine Hospital



Service, to Mr. H. A. Whittaker, chemist and bacteriologist to the Minnesota State Board of Health, and to Mr. James C. Harding. From the report of the latter I have obtained much of my engineering data. Dr. Lumsden's report of the first epidemic has furnished me much valuable material. The description of the installation and operation of the hypochlorite plant has been taken almost verbatim from Mr. Whittaker's report to the Lincoln City Council.

# STUDIES ON THE VIRUS OF HOG CHOLERA.\*

## PRELIMINARY REPORT.

WALTER E. KING AND F. W. BAESLACK.

(From the Research Laboratory, Parks, Davis & Co., Detroit, Mich.)

This preliminary report is presented for the purpose of recording certain observations, which have been made by the aid of the dark field on the blood of hogs suffering from hog cholera. During the last few months a spirochete has been found with uniformity and constancy in the blood of every cholera hog examined. Practically all of these positive findings have been controlled by one or more careful dark field examinations of the blood before inoculation. Additional checks are furnished in several cases by negative findings subsequent to positive results in blood of hogs recovered from the disease.

In this work the fresh blood of normal and diseased hogs is collected aseptically in sterile sodium citrate solution. It is then examined at once with the dark field. The blood of normal hogs thus examined may show, in addition to the normal structures, a few granular bodies, sometimes a few bacterial cells from possible extraneous contamination, and filaments. The latter may assume the form of "dumb bells," "chains," or flexible filaments, which by an untrained observer might easily be mistaken for spirochetes. An excellent description of these bodies as well as others found in the blood of normal animals is given by Balfour.<sup>†</sup>

In so far as the present results go, the practiced observer can readily distinguish certain characters in the blood of animals suffering from hog cholera when placed on the dark field, as differentiated from normal hog blood. It usually contains many granules, some very fine yet distinctly larger than blood dust, some larger still, and some very distinct, highly refractive bodies. It is possible that some or all of these granules represent disintegrated blood elements resulting from the disease.

\* Received for publication November 30, 1912.

† Fourth Report, Wellcome Tropical Research Laboratories.

In the specimens of blood from all infected hogs which have been observed on the dark field, a relatively large spirochete has been found. It averages from five to seven microns in length and one micron in width. The body of the organism is flexible, round and blunt at its ends. It is actively motile and revolves about its longitudinal axis. Its motility is undulating in character and its spirals are fixed. Several of these have been observed dividing longitudinally. In one permanent microscopical mount, prepared by india ink fixation, one of these organisms shows a polar flagellum. This spirochete is readily distinguished from bacteria on account of its lack of rigidity and characteristic motility, and from "blood filaments" by its refractive properties and characteristic morphology.

To date, positive findings of this spirochete are recorded in the following strains of virus:

- 1) Michigan strain of hog cholera virus (originally B.A.I. strain of virus).—In the blood of seven hogs, all checked by dark field examinations of normal blood, except the first one, in which was observed the spirochete.

- 2) Michigan strain (Demerick farm).—In six hogs all checked by normal examinations, except the first two, which were suffering from hog cholera when received from the owner.

- 3) Indiana strain.—In one hog controlled by negative findings in blood 24 hours after inoculation.

- 4) Michigan strain (Rochester).—In seven hogs all of which were showing symptoms when received. In these animals diagnosis was practically made by means of the dark field.

- 5) California strain.—In one hog, checked by repeated examinations of blood before inoculation.

- 6) Kansas strain.—In one hog checked by examination of blood before inoculation.

- 7) Ohio strain (Pettigrew).—In one hog checked by examination of blood before inoculation.

- 8) Unknown (mixed) strain of virus.—In four hogs, infected through natural exposure. These hogs were supposed to be healthy and after obtaining positive findings in the blood of one, physical inspection showed that all were manifesting early clinical symptoms of hog cholera.

9) Controls are furnished by repeated negative findings in two untreated animals kept for a time as checks, by repeated negative findings in four "natural immunes," and in six animals which became convalescent and finally recovered.

To date 33 hogs have been under observation, 130 dark field examinations have been made, and permanent microscopical preparations of the organism in the B.A.I., Indiana, and Rochester strains of virus have been secured.

Two experiments have been made relative to the horse serum virus phenomenon,<sup>1</sup> which showed the presence of the spirochete in the horse serum virus.

The data thus far collected in this investigation are limited and do not warrant the formation of definite conclusions as to the etiological significance of the findings. The details of the experiments and additional material will be presented in a later publication.

<sup>1</sup> King and Wilson, *Jour. Infec. Dis.*, 1912, 11, p. 441.

## A BACILLUS FROM SPONTANEOUS ABSCESES IN RABBITS AND ITS RELATION TO THE INFLUENZA BACILLUS.\*

DAVID J. DAVIS, M.D.

(From the Pathological Laboratory of St. Luke's Hospital, Chicago.)

For several years past, and especially during the last year, there have come under my observation many rabbits with abscesses with certain rather characteristic features. Interest in these abscesses was aroused because a pleomorphic bacillus having some features in common with organisms of the influenza group was found in the pus, usually pure in all cases. Inasmuch as pathogenic hemophilic bacilli have never been found in animals other than man, it was deemed important to study this bacillus somewhat minutely.

The abscesses occur on the abdomen, sides, extremities, neck, and especially on the back and at the base of the ears. They may be single but often are multiple, there being at times six or eight distinct abscesses in a cluster. They always occur in the loose subcutaneous tissue and do not invade the subjacent structures. They are at first free from the skin, but later they may break down and discharge their contents. They are distinctly encapsulated and vary in size from that of a pea to that of an orange or larger. If incised and evacuated they usually disappear. The animals at first appear healthy but later usually show some emaciation which may become extreme if the abscesses grow to large size. The capsule or wall of the abscess is usually 1-3 mm. thick and consists of connective tissue which is easily ruptured. The content early is highly purulent and quite moist; later it becomes yellowish gray, dry, and cheesy in appearance. It may have a rancid, disagreeable odor. Stained smears reveal leukocytes in all stages of disintegration, much granular fatty detritus and bacteria.

*Bacteriological examination.*—Abscesses from 18 animals have been examined and in each there was found both in smear and culture a characteristic bacillus. As a rule it was in pure culture but in some abscesses which had previously ruptured staphylococci were mixed with it. In smears made directly from the pus the bacillus is usually rather long, irregular, often arranged in pairs end to end and sometimes in longer chains of four, six, or eight elements. The ends are rounded and at times in older pus the organism may be thread-like and tortuous. It is nearly always found outside the leukocytes. In cultures it often loses its tendency to form long threads, at least to a considerable extent. Consequently it appears usually as a small bacillus with here and there filaments of different lengths. Some strains are very small, being

\* Received for publication November 21, 1912.

about the size of influenza bacilli but more coccoid. The thread-like filaments also resemble closely those seen in cultures of the influenza bacillus. While the size varies greatly, the average length may be given as  $1-1.2 \mu$  and the width as  $0.3-0.4 \mu$ .

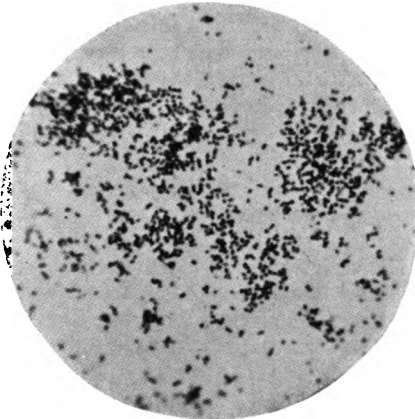


FIG. 1.—Pure culture of bacillus isolated from an abscess in a rabbit. Little or no tendency to form threads. The bacilli are about the size of the influenza bacillus.  $\times 1,000$ .



FIG. 2.—Pure culture of same strain as shown in Fig. 1, after growing for some time on artificial media.  $\times 1,000$ .

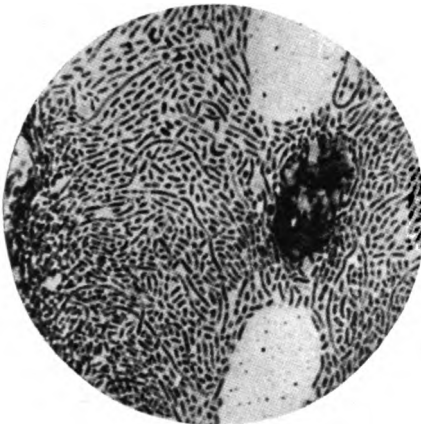


FIG. 3.—Pure culture of abscess bacillus, showing marked tendency to form filaments. A disintegrating leukocyte is seen to the right.  $\times 1,000$ .

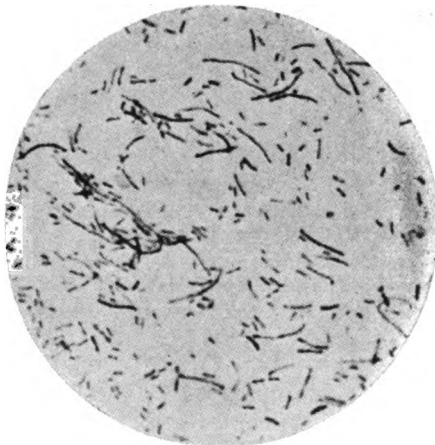


FIG. 4.—Pure culture of so-called pseudoinfluenza bacillus, a strictly hemophilic organism. Note similarity to organism shown in Fig. 2.  $\times 1,200$ .

In the hanging drop no definite grouping is apparent beyond that mentioned above. There is no motility, and spores and capsules have not been noted. It stains readily with ordinary dyes, the ends, as a rule, taking the stain more intensely than the middle portions. Distinct granules have not been seen in the bacillus. It destains with

gram, though perhaps with somewhat more difficulty than the influenza bacillus. It is non-acid fast.

On plain agar the growth often fails altogether when the organism is first isolated. After several generations it may become more profuse. It is gray, moist, flat, slightly spreading, translucent, and distinctly viscid; no odor and no chromogenesis.

Blood agar is by far the most favorable medium. Growth is abundant in 24 hours, quite viscid, and with the characteristics noted on plain media; no hemolysis. On serum agar and Loeffler's blood serum growth is fairly abundant, though when first isolated, the bacillus may grow very feebly or fail to grow altogether. After growing on blood agar for a time the growth on serum media may be quite as profuse as on blood media. No liquefaction of the serum media.

In agar stab cultures growth occurs along the entire needle track, best at the top but with scanty surface growth. Glycerin agar yields a more profuse growth than plain agar. Gelatin stab reveals scanty growth along the needle track and without

liquefaction. On potato as a rule there is no growth or only the merest trace; no pigment. In broth there occurs moderate turbidity without pellicle and with slight sedimentation; no odor. In litmus milk no change occurs; growth is doubtful.

The individual colonies are best observed on blood-agar plates. In 24 hours they may attain the diameter of 1-2 mm. They are grayish blue, translucent, slimy, flat, and non-hemolytic. With a low power the colonies have entire margins, are finely granular and round or slightly oval in shape.

Indol is not produced and gas is not formed in glucose media. The bacilli live from two to three weeks at room temperature in culture tubes

and are killed in 15 minutes at 60° C., but not at 55° C. for 30 minutes. They grow slowly at room temperature and best at 37° C.

While the observations in connection with these abscesses were going on there was noted among the animals an occasional case of pleuropneumonia. During the last year this disease became epidemic and caused the death of nearly all the animals in the laboratory at the time. This disease, known also by some as rabbit influenza, snuffles, etc., appears to be not uncommon in laboratories and probably may be caused by a number of different germs, as will be pointed out later. As regards these cases the onset was usually manifested by a profuse discharge from the nose. More

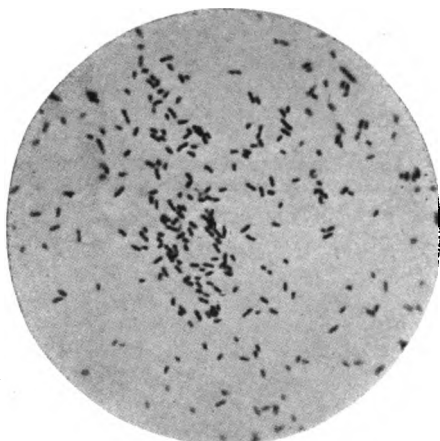


FIG. 5.—Influenza bacillus; pure culture. Compare with Fig. 1.  $\times 1,200$ .

than half of the infected animals died in the course of a few days. When not fatal, recovery took place in from 10 days to two weeks. Such animals appeared to be immune to natural infection, though they were susceptible to inoculations with cultures of the bacilli. At autopsy invariably there was found a rich fibrino-purulent exudate in one or both pleural cavities and a pneumonia involving portions of a lobe or at times the entire lobe. The mucosa of the upper respiratory tract was always red and swollen. Microscopically the exudate was rich in cells. Fibrin in the alveoli and the bronchi was abundant and the parenchyma was deeply infiltrated with inflammatory cells. At times a sero-fibrinous pericarditis and also peritonitis existed. From the exudates and from the heart's blood, in every case without exception, there was isolated a small bacillus.

This bacillus is a gram negative, non-motile, polar staining minute bacillus which does not form spores and is not hemolytic. In smear preparations it is usually short and rather plump, but nearly every strain examined showed some large thread forms. In some strains they were common, in others scarce. The morphology of this bacillus together with the observation that it at times failed to grow on media not containing blood not only suggested a relation to the influenza bacillus but also suggested a possible relationship to the bacillus which had been isolated from the abscesses. A comparative study was therefore made of a number of strains isolated from animals dying of pleuro-pneumonia and strains obtained from abscesses.

Briefly, on the various culture media no essential differences were noted. Slight variations at times appeared, but only such as might occur between various strains of the same organism. On milk, potato, broth, blood agar, serum media, etc., they grew exactly alike and on various sugars they could not be differentiated. They all fermented glucose, saccharose, and mannite without gas formation and produced no change in lactose, raffinose, and maltose.

*Pathogenicity.*—These bacilli are invariably pathogenic for rabbits and guinea-pigs. A suspension of the bacillus from the abscesses inoculated subcutaneously into rabbits will produce lesions identical with those occurring naturally. The abscesses grow slowly and after weeks or months may attain enormous size. In the case of one animal which lived five months an oblong abscess developed at the point of inoculation



in the back, growing slowly but gradually and at the time of death measured 16 cm. in length, 8 cm. in width, and 6 cm. in depth. The animal became greatly emaciated. From the cheesy contents of the abscess the bacillus was recovered in pure culture. The heart's blood was sterile and the organs showed no important alterations. No other abscesses occurred anywhere in the body. Two rabbits were inoculated intratracheally with bacilli isolated from the abscess. One animal died a few hours later without any specific alterations in the organs. The second animal survived one week, at which time it was killed and a distinct region of bronchopneumonia nearly one centimeter across was found in the upper lobe of the right lung. In the bronchi was a small amount of purulent exudate from which the bacilli were recovered. Guinea-pigs appear to be more susceptible. Intraperitoneal injections kill in from one to three days and the organisms are found in the heart's blood and body cavities. Subcutaneously a small local abscess with edema and necrosis usually develops which may become walled off and heal or may kill by general infection. Large chronic abscesses such as occur in rabbits do not appear to develop in guinea-pigs.

The bacilli isolated from the pleuropneumonia when inoculated subcutaneously in small doses into rabbits will cause chronic abscesses in every respect like those caused by bacilli isolated from the abscesses. One drop or less of broth culture is sufficient for this purpose. If a much larger quantity is given the infection rapidly spreads in the subcutaneous tissue accompanied by intense edema and necrosis and the animal dies very soon of septicemia. In such experimental infections there may be involvement of the lungs in the form of typical pleuropneumonia, but often there is not. A number of rabbits were inoculated intravenously, and in all death ensued within 24 hours without localization. The bacilli multiplied very rapidly in the blood and appeared in large numbers in smears from the heart. The bacilli from bronchopneumonia injected into guinea-pigs subcutaneously or intraperitoneally produce lesions identical with those caused by the abscess bacillus.

Some further observations were made bearing on the pathogenicity of these bacilli and also on the possible origin of the subcutaneous abscesses. I think there is little doubt that they arise from bites and scratches received from other rabbits, though in most instances this cannot be definitely determined because the skin over the abscesses shows no ulceration or scars. The following observation was made: In the case of a large male rabbit, fatal infection resulted following a bite in the side received from another male rabbit during a fight. A large tear in the skin was found, and at this point there resulted a local acute infection with extensive edema. This infection extended along one of the large veins on the animal's side up to the subclavian vein, causing an extensive thrombosis. A generalized infection resulted in death, and from the heart's blood, peritoneum, and the lesion on the side the bacillus was obtained in pure culture. It is to be noted, however, that in this animal watery discharge from the nose and pleuropneumonia were not present. As a control to this observation a large rabbit was inoculated in the side, at approximately the same point as the above rabbit was bitten, with a large dose (one broth culture) of a strain isolated originally from a typical chronic abscess. At the site of injection there appeared a few days later extensive infiltration soon followed by death of the animal. From the infiltrated region thrombosis of the veins extended to the subclavian vein and into the heart exactly as occurred in the animal that was bitten.

From the above experiments it would seem probable that the abscesses arise from bites or scratches. The rabbit which inflicted the bite, it may be stated, was normal

in every way, though other animals in the laboratory at the time were suffering with abscesses, but none with pleuropneumonia.

There can be no doubt, then, that the bacillus found in the abscesses is the specific cause of these lesions, since typical lesions can be produced with pure cultures and from such lesions the organisms can be again recovered. The bacillus found in the lesions of pleuropneumonia is undoubtedly the specific cause of this disease also. Furthermore, the bacillus of pleuropneumonia may cause typical chronic abscesses in rabbits if small doses are given. But the results of inoculation of animals with the abscess bacillus even in large doses cannot be said to reproduce, in every respect, the clinical and pathological picture of pleuropneumonia. A pneumonia was experimentally produced by intratracheal injection, but this might be expected with almost any organism. I am inclined to believe, in view of the fact that the organisms are practically identical culturally and in other respects, that the abscess bacillus is the same organism as the pneumonia bacillus but reduced in virulence. They may, however, be closely related organisms of the same group.

In order to test this point, agglutination experiments were made with the serum of animals suffering with the disease. The serums from four rabbits which had abscesses for periods of from one to four months were tested, using both autogenous bacilli and strains which had been isolated from other animals as well as strains from cases of pleuropneumonia. Not a trace of agglutination was found in any instance. Furthermore two guinea-pigs were treated with dead bacilli and after several injections no trace of agglutinin was found in the animals. The serum from animals with pleuropneumonia likewise failed absolutely to agglutinate the bacillus causing the disease.

From these results it cannot perhaps be definitely decided whether or not the bacillus from the abscesses and that causing pleuropneumonia are identical, but they certainly appear to be very similar, and probably they are one and the same organism.

Concerning the relation of these bacilli to the influenza bacillus, morphologically they are quite similar and might readily be confused. The peculiar thread formation in both is much the same

and the character of the infection of the respiratory tract in rabbits might be considered suggestive as is indicated by the fact that the disease is sometimes known as "rabbit influenza." However, these bacilli, though preferring media containing blood, are not strictly hemophilic, as are the influenza bacilli, and they show no evidence of symbiosis which is another very distinctive property of true influenza bacilli. They should not be classed therefore in the influenza group.

From a study of the literature there is some doubt whether this organism from abscesses has been previously described. Schimmelbusch and Mühsam<sup>1</sup> in 1896 described an organism isolated from abscesses in rabbits which in many respects agrees with the bacillus here described and probably is identical, though their somewhat incomplete description does not permit a satisfactory comparison. Their organism was a gram negative, non-motile, short bacillus with many of the cultural features of the bacillus here described and which upon reinoculation produced similar chronic abscesses. Lexer<sup>2</sup> used this organism in certain experiments designed to produce osteomyelitis in rabbits. He found that when the bacillus alone was inoculated intravenously no results were obtained; but mixed with staphylococci abscesses were produced in bone which the cocci alone would not cause. Also, he found that if the bacillus was injected three days after a staphylococcus injection, multiple joint infection would result from which lesions the bacillus could be recovered mixed with the staphylococcus or at times pure. Lexer points out the possibility of a symbiotic relationship existing between these organisms.

In the literature are reports also of a number of varieties of bacilli which have been isolated from rabbits dying of pleuropneumonia (*Brustseuche*), but though all of these bacilli resemble each other at least in certain respects, many differ to such an extent that they cannot be considered identical.

Beck<sup>3</sup> in his discussion of pseudoinfluenza bacilli mentions a small, gram negative, non-motile bacillus, pathogenic for rabbits,

<sup>1</sup> *Arch. f. klin. Chir.*, 1896, 52, p. 564.

<sup>2</sup> *Ibid.*, p. 576.

<sup>3</sup> *Handbuch der path. Micro.*, 1903, 3, p. 405.

guinea-pigs, and mice and having a marked tendency to form threads, as the cause of *Brustseuche* in rabbits, in which condition fatal bronchopneumonia and pleurisy occur. This organism is somewhat similar to the influenza bacillus or rather the pseudo-influenza bacillus. Schimmelbusch and Mühsam discuss Beck's organism and think it slightly different from the bacillus they found in the abscesses. They point out especially that Beck's organism forms threads like the influenza bacillus, whereas their organism never does. The bacillus here described often formed filaments, in this respect more closely resembling Beck's bacillus. But neither are strictly hemophilic, nor do they show the property of symbiosis; consequently they differ from the influenza bacillus in two of its most definite and characteristic properties and should not be included in the influenza group.

Laven<sup>1</sup> described a bacillus pathogenic for rabbits and guinea-pigs which he said had been heretofore undescribed. He isolated it from an animal dying of *Brustseuche* and also from abscesses appearing on rabbits which seem to have been quite similar to the abscesses described in this paper. It is a small gram negative bacillus tending to grow in chains and threads, and is variable in size. According to the description given it differs in a number of important points from the bacillus I describe. For example, it is slightly hemolytic, it gives a peculiar sperm-like odor on blood agar, and does not form acid in dextrose, saccharose, and mannite. Morphologically the two closely resemble each other.

Recently Glaue<sup>2</sup> also reports finding a small bacillus as the cause of an epidemic of *Brustseuche* in rabbits. This disease was evidently quite different from the one here described. There was never any running at the nose in the affected animals which was such a striking characteristic in our epidemic. The bacillus never formed threads and was peculiar in that the growth took on a characteristic dry appearance after 48 hours, a point to which he called especial attention. This latter point was never observed in our cultures. He does not mention its relation to abscess production in rabbits. He contends that his bacillus is different from

<sup>1</sup> *Centralbl. f. Bakt., I, Orig.* 1910, 54, p. 97.

<sup>2</sup> *Ibid.*, 1911, 60, p. 176.

Laven's bacillus morphologically, culturally, and in its pathogenic effects. It was not hemolytic and growth on blood agar was not more abundant than on media without blood.

Kurita<sup>1</sup> described a small, gram negative, polar staining bacillus which when first isolated grew only on blood or egg media, but later would grow fairly well on plain media. He isolated this bacillus from rabbits dying of septicemia and it was pathogenic for rabbits, guinea-pigs, and mice. It killed rabbits when injected intraperitoneally or intravenously by producing *Brustseuche*, and when injected subcutaneously produced abscesses in both guinea-pigs and rabbits. He believes his bacillus is different from Beck's bacillus.

None of these bacilli correspond exactly with the bacillus described in this paper nor are any two of them exactly alike. Laven has tabulated the various germs of this group which have been found in rabbits and guinea-pigs and for detailed differences reference may be made to this paper. Including his own, 14 gram negative organisms have been described. Four are motile and one of the number liquefies gelatin. Though they all produce similar pathologic lesions in the infected animals, in many other respects they are quite different. The organisms which most closely resemble my bacillus have been referred to and pointed out above. It is to be noted that with the exception of the work of Schimmelbusch and Mühsam all of the reports deal with the relation of these bacilli to lesions of the respiratory tract. The relation between the subcutaneous abscesses and the respiratory lesions apparently has not been noted and for this reason I have called particular attention to this phase of the subject.

#### SUMMARY.

Spontaneous subcutaneous abscesses in rabbits are caused by a definite bacillus for which all of Koch's postulates have been fulfilled. At times the bacillus produces acute fatal infections in animals following bites. Bites or scratches are probably the common mode of infection.

<sup>1</sup> *Centralbl. f. Bakt.*, I, Orig. 1909, 49, p. 508.

The bacillus is pleomorphic, tends to form threads and non-branching filaments, but is not strictly hemophilic, nor does it manifest the phenomenon of symbiosis in cultures. It should not be classed in the influenza group.

This bacillus is identical culturally and in many respects morphologically with a bacillus which caused an epidemic of pleuropneumonia in rabbits. In small doses the latter produces subcutaneous abscesses similar in every respect to those caused by the abscess bacillus. The abscess bacillus by intratracheal injection may cause pneumonia.

Agglutinins have not been noted in the sera of infected animals.

## GENERAL GONOCOCCUS INFECTION IN A MALE CHILD WITHOUT EVIDENCE OF URETHRITIS.\*

MATTHIAS NICOLL, JR., AND M. A. WILSON.

(From the Research Laboratory, Department of Health, New York City.)

The patient, a boy aged two and one-half years, was admitted to the Scarlet Fever Hospital of the Department of Health on the service of Dr. Alfred Hess on June 14. He gave an indefinite history of scarlet fever three weeks previously.

*Physical examination.*—No eruption on body. Desquamation on palms and soles. General anasarca. Face and eyelids very puffy. Heart: soft, systolic whiff over the aortic area. Second sound accentuated. Area of cardiac dulness increased three centimeters to the left. Lungs: Slightly high-pitched breathing over the left apex in front. Temperature: 100° F., pulse 170, respiration 22. Urine: Sp. Gr. 1022, acid, marked trace of albumin, many granular casts, much free blood. The patient was very apathetic.

*Subsequent condition.*—During the next few days the condition of the kidneys showed marked improvement under diuretic and diaphoretic treatment. The pulse, however, continued to be very rapid.

June 27, the left elbow and knee became swollen and very tender, evidently containing fluid. This was preceded for several days by a marked rise of temperature, 102° to 103° F. The condition was regarded as due to a scarlatinal arthritis. During the next two weeks the affected joints continued to be in practically the same condition. The pulse was rapid, the afternoon temperature 101° to 102°. The patient was extremely emaciated.

July 17, there was an exudation on both tonsils and posterior pharynx. Cultures showed diphtheria bacilli. Ten thousand units of antitoxin were given. This condition cleared rapidly. There was a swelling, the size of a hen's egg, over the right sterno-clavicular articulation, which developed within a few days into an abscess from which pus, aspirated under conditions to prevent contamination, was sent to the laboratory for examination. Gonococci were found to be present. The abscess was then opened and drained. The temperature and pulse, which had risen during the formation of the abscess, fell somewhat after the operation. A blood culture taken on this day to determine if general sepsis was present proved negative.

July 24, the left shoulder was swollen and painful. It was incised and drained five days later, a quantity of thick pus evacuated and sent to the laboratory for examination, and gonococci found.

August 4, as the presence of gonococci was definitely determined in the abscesses, vaccine treatment was begun, 100,000,000 of a polyvalent preparation being given.

August 9, a blood culture to determine the presence of gonococci proved negative. At the time of beginning specific treatment the open wound in the sterno-clavicular regions was practically healed. The opening about the left shoulder joint showed

\* Received for publication October 17, 1912.

flabby granulations and much discharge. The left knee joint was greatly swollen and contained fluid. It was exceedingly tender to the touch or on attempting passive movement. The leg was held stiffly at right angles. The left elbow joint showed practically an identical condition. The patient was greatly emaciated. A week after beginning treatment the local and general condition began to improve rapidly, the swelling and tenderness growing less from day to day and the patient began to eat and gain flesh. The course of vaccines is indicated on the temperature chart.

At the time of his discharge from the hospital on August 24 he was fairly well nourished and seemed well. The involved joints were moderately thickened. There was little or no pain on active or passive movement. There was, however, in the elbow joint marked crepitation on passive movement, probably an evidence of some

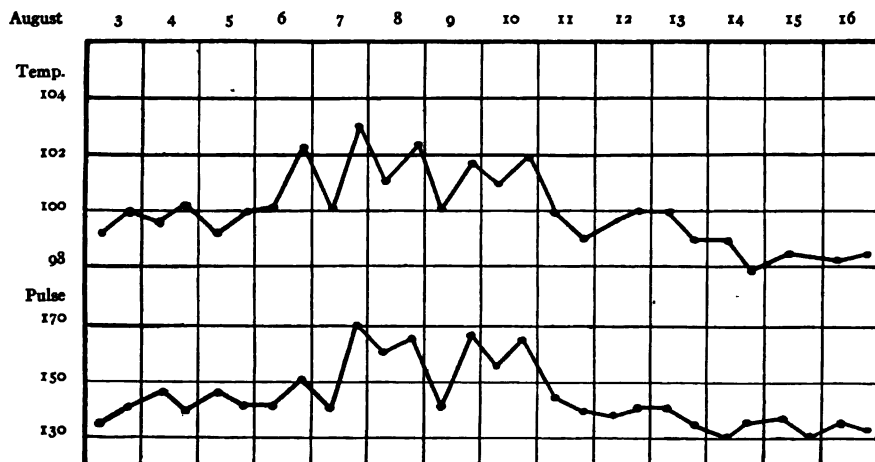


CHART 1.—Temperature and Pulse Curves.  
Vaccine administered August 4, 7, 9, and 14 in doses of 100 million, 100 million, 150 million, and 200 million strength respectively.  
Blood culture made August 9.

permanent damage. The wound in the shoulder had been completely healed for some 10 days. There was no apparent abnormality to be made out in the heart although the pulse rate continued above normal.

During the patient's stay in the hospital a number of small abscesses developed about the face and extremities. Cultures from them showed only staphylococci, and they healed readily after incisions. A circumscribed necrosis opposite two teeth in the lower jaw yielded rather slowly to local applications. Smears from the necrotic area showed mixed bacteria with many fusiform bacilli and spirochaetes.

There had never been the slightest evidence of urethritis as shown by discharge or difficulty in urination during the patient's stay in the hospital nor had the parents noted any such symptoms before his admission. With the object, however, of determining the point of entry of the invading organism, a number of urethral cultures were made together with one from the tonsils. The latter proved negative. The results of the former together with those from the affected joints are as follows:



*Urethra*.—1st culture—unsatisfactory, negative.

2d culture—gave a gram negative bacillus.

3d culture—on ascitic plates showed typical gonococcus colonies, fishings from which gave gram negative cocci morphologically identical with gonococci.

4th culture—negative.

5th culture—smear from swab showed intracellular gram negative diplococci, cultures from swab on ascitic agar plates gave gonococcus colonies after 24 hours, fishings from which showed a pure culture of morphologically typical gonococci.

*Sterno-clavicular Joint*.—Cultures showed a symbiotic growth of gram negative diplococci and gram negative bacilli; the former could not be isolated in pure cultures.

*Shoulder*.—Smears from the pus showed intracellular gram negative diplococci. Blood-streaked agar plates after 48 hours showed typical gonococcus colonies, fishings from which gave morphologically typical gonococci in pure culture.

*Complement fixation*.—Pure cultures of the gram negative cocci from the shoulder and urethra autolyzed at 56° in distilled water for 24 hours used as an antigen and tested against gonococcus immune rabbit's and horse's blood gave in each instance a high degree of fixation.

#### CONCLUSIONS.

1. Although two blood cultures failed to show the gonococcus, it is evident that the blood stream carried this organism to the widely distant points of localized infection.

2. The gram negative bacillus found in symbiosis with the gram negative coccus in the sterno-clavicular joint was in all probability identical with that found in the urethra and carried from it to the joint together with the gonococcus.

3. The fact that two of the joints did not suppurate is no reason for regarding their condition as due to the invasion of other organisms than the gonococcus, especially in view of the promptly beneficial action on them produced by specific treatment.

4. The presence of virulent gonococci in the male urethra does not necessarily cause recognizable local symptoms.

## ON THE CULTIVATION OF THE TREPONEMA PALLIDUM (SPIROCHAETA PALLIDA).\*†

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Attempts to cultivate the *treponema pallidum* in artificial media followed closely on the discovery that this organism is the cause of syphilis. Volpino and Fontana<sup>1</sup> reported in 1906 on the cultivation of the organism in serum-agar. The observations of Volpino and Fontana were corroborated by Schereschewsky<sup>2</sup> in 1909. By inserting deep into coagulated horse serum small pieces of human tissue from syphilitic lesions, he obtained impure cultures. The horse serum was liquefied around the tissue, and in this he was able to find the spirochaetes. These cultures he was able to transfer. Mühlens<sup>3</sup> in 1910, made use of the same medium, as did also Hoffmann,<sup>4</sup> in 1911, and both were able to obtain cultures of the organism. These cultures also were contaminated with bacteria introduced with the tissue used for starting the cultures. For the purpose of purifying these cultures they employed serum-agar. The attempts of these investigators to produce syphilitic lesions through the injection of cultivated spirochaetes failed for a time, probably because the dose employed was too small. Both Schereschewsky and Mühlens concluded that the strains which they had cultivated were non-pathogenic.

Brückner and Galascesco<sup>5</sup> in 1910, as well as Sowade<sup>6</sup> in 1911, claimed to have succeeded in producing syphilitic lesions in rabbits by the injections of large quantities of impure cultures grown on coagulated horse serum and ascitic fluid media still containing the original syphilitic tissue. The failure to grow again the spiro-

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<sup>1</sup> *Deutsch. med. Wchnschr.*, 1909, 35, p. 835.

<sup>2</sup> *Zeitschr. f. Hyg. u. Infekt.*, 1911, 68, p. 27.

<sup>3</sup> *Ibid.*, p. 1260.

<sup>4</sup> *Compt. rend. soc. d. Biol.*, 1910, 68, p. 684.

<sup>5</sup> *Ibid.*, p. 1261; *Klin. Jarb.*, 1910, 23, p. 339.

<sup>6</sup> *Deutsch. med. Wchnschr.*, 1911, 37, p. 682.

chaetes from the testicular lesions produced, lays the results claimed by these investigators somewhat open to question.

Noguchi<sup>1</sup> in 1911 published a method for the pure cultivation of pathogenic *treponema pallidum*. Instead of attempting to obtain a pure culture of the organism through cultivation in artificial media, he first transplanted the human syphilitic tissue into the testicles of rabbits. This procedure, if carefully done, so as to avoid accidental contamination, will in a few passages through rabbits clear the strain of spirochaetes from the accompanying infection so that in a comparatively short time lesions may be produced in rabbits which contain the organisms in pure form. These Noguchi used for cultivation experiments. After many unsuccessful attempts with various media he found that inactivated serum-water was most suitable for the purpose. In order to obtain strict anaerobiosis he placed a small piece of normal rabbit's tissue, either kidney or testis, at the bottom of the medium. He found it necessary to place the culture under hydrogen with pyrogallic acid and potassium hydroxid. The spirochaetes thus grown could be gradually transferred to solid media, i.e., ascitic-agar plus tissue. These cultures were pathogenic for rabbits. This announcement of the successful inoculation of rabbits with the *treponema pallidum* grown in culture was followed, within a month, by that of Hoffmann<sup>2</sup> and Schereschewsky<sup>3</sup> who had succeeded also in producing syphilitic lesions in rabbits through the injection of pure cultures of this organism.

Since then numerous investigators have taken up the cultivation and study of the *treponema pallidum*. Aside from those already mentioned Leuriaux et v. Geets,<sup>4</sup> Tomaszewski,<sup>5</sup> Sowade,<sup>6</sup> Arnheim,<sup>7</sup> and others have succeeded in growing this organism.

In this country Noguchi seems to be the only investigator, who has reported on the cultivation of the *treponema pallidum*. The reason for this lack of confirmatory work may lie in the rather difficult method which he uses.

<sup>1</sup> *Jour. Exper. Med.*, 1911, 2, 14, p. 99; *München. med. Wchnschr.*, 1911, 29, p. 1550.

<sup>2</sup> *Menschen. deutsch. med. Wchnschr.*, 1911, 34, p. 1546.

<sup>3</sup> *Ibid.*, 1911, 39, p. 1708; *ibid.*, 1912, 28, p. 1335.

<sup>4</sup> *Centralbl. f. Bakt.*, 1912, 61, p. 684.

<sup>5</sup> *Deutsch. med. Wchnschr.*, 1912, 17, p. 797.

<sup>6</sup> *Berl. klin. Wchnschr.*, 1912, 17, p. 792.

<sup>7</sup> *Ibid.*, 1912, 20, p. 934.

The media described by Noguchi as most suitable for the cultivation of the *treponema pallidum* offer many difficulties and demand extraordinary care. The solid media have to be prepared fresh for each transplantation, for it is necessary that the tissue, placed in the bottom of the tube, be fresh. Furthermore, the use of neutral oil on the top of the media increases the difficulty of making examinations from time to time. Noguchi also pointed out that not all lots of ascitic fluid are suitable for the ascitic-agar. Having tried many without success, until I finally found one, I faced the same difficulty again after that was used up. For these reasons it seemed worth while to test also the methods used by other investigators, since these methods are simpler and the materials used for the making of the media more constant and easier to obtain.

Probably the fact that the investigators abroad started their cultures directly from the syphilitic lesion of man, whereas Noguchi at first made use of material obtained by passing the *treponema pallidum* through rabbits, brought about a divergence in the interpretation of the cultural characteristics of this organism and cast doubt upon the genuineness of the cultures in the possession of some of the investigators. Thus Noguchi maintains that the following conditions are essential to the growth of the *treponema pallidum*: (1) the presence of suitable fresh sterile tissue in serum-water, (2) strict anaerobiosis (3) a slightly alkaline reaction as furnished by the serum and tissue, and (4) a temperature of about 35°-37° C. Noguchi further states: "The presence of agar or gelatin seems to interfere with the successful growth of the first generation of the spirochaeta." Arnheim, cultivating the *treponema pallidum* directly from the human syphilitic lesion, plants his material into serum-agar without tissue. Nakano<sup>1</sup> cultivates the *treponema pallidum* in sterile horse serum without the addition of tissue and the strict anaerobiosis, using a rubber stopper only to exclude air. Schereschewsky and Sowade make use of the same technic as Arnheim. Their cultures were obtained without the addition of sterile tissue, or strict anaerobiosis. Furthermore, Schereschewsky<sup>2</sup> observed that the *treponema pallidum* will grow well at a temperature of 40° C. It is also

<sup>1</sup> *Deutsch. med. Wchnschr.*, 1912, 28, p. 1334.

<sup>2</sup> *Ibid.*, 1912, 28, p. 1335.

interesting to note that Noguchi has been able to grow the *treponema pallidum* without the hydrogen, simply placing a layer of sterile neutral oil on top of his media. This diversity of conditions under which these cultures of the *treponema pallidum* were grown may be due to the fact that on the one hand the cultures were started from material which has been purified by the passage through rabbits, while on the other hand the cultures were obtained directly from human lesions rich in the *treponema*.

My attempts to cultivate this organism were begun in the fall of 1911, and efforts were first directed to the inoculation of rabbits in order to obtain material rich in the *treponema* and possibly sufficiently pure from banal bacteria to attempt cultivation. The unpleasant odor of the cultures, as well as the liquefaction of the culture medium, described as characteristic of the *treponema* culture by some of the earlier investigators, seemed to me to be due to the concurrent infection and not due to the *treponema pallidum*. Another reason why I transplanted first into rabbits before attempting to inoculate culture media, was the fact that the opportunity for obtaining syphilitic tissue containing the *treponema* is relatively limited. It was necessary to use the serum which was drawn from the primary sore by means of a specially devised cup and suction pump. The small amount of serum thus obtained was employed for diagnostic purposes as well as for the inoculation of rabbits.

The apparatus for obtaining the serum consists of a test tube drawn to a fine point, with a side arm for the attachment of the rubber tube, which in turn is connected to a small suction pump. The tube has a fairly large lip, which allows it to fit snugly on the tissue surrounding the primary lesion. I use cupping tubes varying in diameter to accommodate chancres of different sizes. The pump is manipulated by means of a crank and has a screw on its lower end, which when loosened will allow air to enter. This permits of releasing the vacuum in the cupping tube, and does away with any pain which the removal of the tube might cause (Fig. 1).

The inoculation of the rabbit is made with a glass hypodermic syringe in such a way that a small depot of the serum is created in the testicle of the rabbit. With this method I have obtained

two strains of the *treponema pallidum*. The period of incubation following the injection of serum is from eight to ten weeks. The lesions produced at that time were the size of a pea and contained in both instances numerous actively motile spirochaetes. Since then I have succeeded in growing four strains of the *treponema pallidum*. Of these strains, the first, second, and fourth were obtained through the implantation of small pieces of genital chancre tissue into the testicles of rabbits, the third strain was obtained by the injection into the testicle of a rabbit of a small

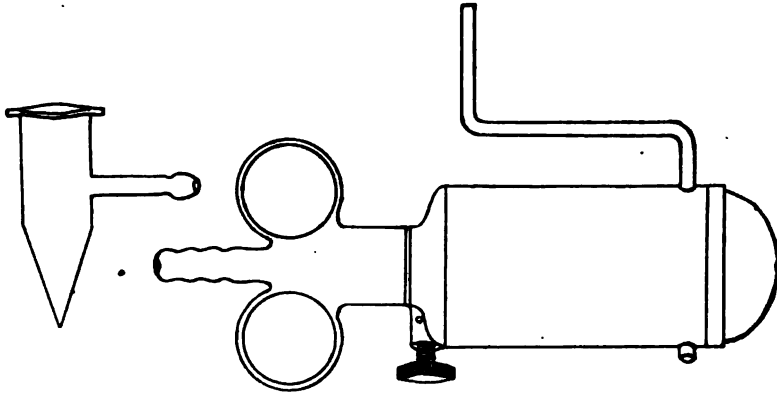


FIG. 1.—Apparatus for obtaining serum.

quantity of serum from another initial lesion. The first strain obtained by the injection of serum obtained by cupping was lost, because the rabbits inoculated in the second generation died of an intercurrent infection. Strains one and two were grown from material supplied by Dr. F. M. Hartsock, whose generous assistance at this stage of the work I gratefully acknowledge.

The transplantations from rabbit to rabbit are carried out as follows. A rabbit with a good-sized unbroken testicular lesion is anesthetized, the lower portion of the abdomen as well as the scrotal region are shaved and sterilized with germicidal soap and alcohol. The rabbit is covered, except the field of operation. The testicle is then removed under aseptic precautions and placed in a petri dish, which is kept warm. To prevent drying, a few drops of sterile broth are added. When the operation is completed the

testis is cut open and the white glistening gumma is cut free from the unaffected part of the testis and placed in another petri dish containing a small amount of broth. The gumma is then cut into small pieces, and a small quantity of the tissue scraping is examined under the dark field to determine the presence of the *treponema pallidum*. A few smears are stained with methylene blue to determine whether the tissue to be used is free from other organisms. To make doubly sure tubes of plain broth and agar are inoculated. The rabbits are then inoculated by means of a small trocar. The inoculation with the trocar has proven more successful than the injection of ground-up tissue. The organisms are not injured to such an extent when solid pieces are introduced, as when the material is subjected to a process of grinding and admixing with a diluent. (I also wish to express my thanks to Drs. Keene, Varney, and Wollenberg for aiding me in obtaining material for this work.) The area of inoculation is cleaned with 70 per cent alcohol and the instruments are boiled for the inoculations of each rabbit. This procedure has the two advantages which Noguchi has pointed out: (1) it enables one to obtain large quantities of spirochaete-containing material at almost any time; (2) the spirochaetes are freed from concurrent microorganisms.

The lesions observed in rabbits vary considerably in severity, general appearance, and location. The most common lesion observed in my work of transplantation of syphilitic tissue in rabbits is a syphilitic orchitis which affects part of the testis only. The scrotum shows no ulceration or lesion or orchitis. The testis is of uniform consistency, except the portion involved by the circumscribed gumma. The lesion is pure glistening white. The fluid, which adheres to the knife on cutting into the lesion, is of a stringy mucoid character, and contains numerous spirochaetes. Such an orchitis may involve the entire testis. The testicle involved is considerably larger than normal.

The second form of syphilitic lesion appears in the form of small nodules or thickenings, which are situated in the tunica. This form of lesion may be circumscribed or it may involve almost the entire tunica. The testis in this case appears small. These nodules and thickenings contain numerous spirochaetes.

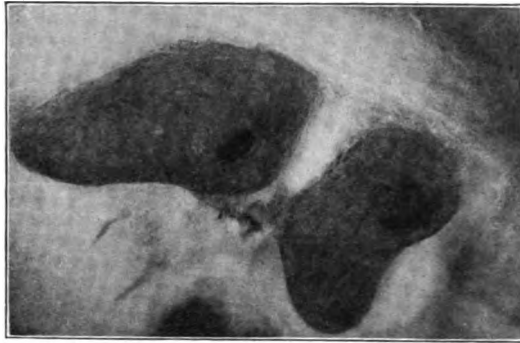


FIG. 2.—Syphilitic orchitis in a rabbit, fifth generation of strain one.

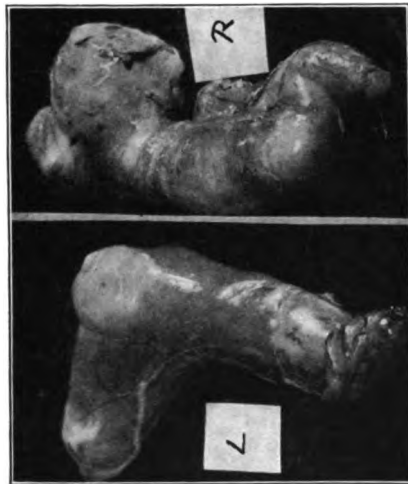


FIG. 3.—The testes of the same rabbit. The lesions may be recognized as white protruding masses.

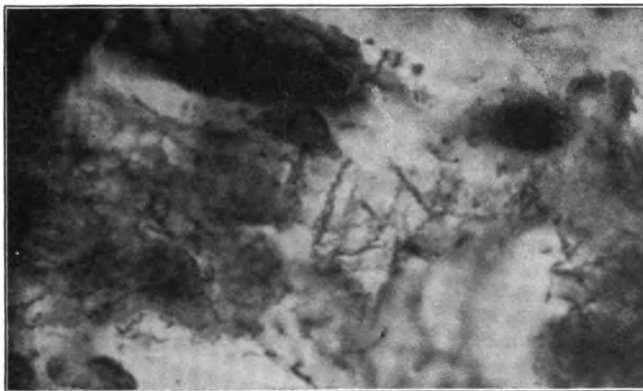


FIG. 4.—Testicular lesion of the rabbit, impregnated with silver, according to Levaditi.



The third form is the ulcer. This varies in size from a few millimeters to a centimeter and even more in diameter. The edges are indurated and the ulcer has the characteristic punched-out appearance. In this form the spirochaetes may be found in the indurated area around the ulcer. The size of the testis is usually not affected by this form of syphilitic lesion.

These varieties of lesions are possibly due to the technic of inoculation, since the tissue frequently adheres to the trocar, is pulled out toward the scrotum, and so is lodged in the tunica, or scrotal skin. The forms described are localized conditions, which in rabbits tend to heal. We have never observed generalized syphilis nor any secondary manifestations.

Soon after the strains 1 and 2 had been passed through rabbits a number of times, attempts were made to cultivate the organism. The cultivation of the *treponema pallidum* demands patience until a difficult technic is fairly well mastered. It is intended to describe the methods employed in detail so that anyone having the necessary laboratory facilities may grow the organism.

The plan followed in my experiments has been to determine whether the *treponema pallidum* can be grown both according to the methods of Schereschewsky, Mühlens, Hoffmann, and according to the method described by Noguchi, thus reconciling the contradictory views regarding the genuineness of the cultures claimed by some of the investigators.

The media employed by Mühlens and Hoffmann are coagulated horse serum and serum-agar. Sterile test tubes are filled with fresh horse serum free from any preservative, and these are sterilized in a water bath for one hour at 60° C. for three successive days. On the third day the temperature is allowed to rise gradually to 70° C. The horse serum will become of a jelly-like consistency and will be transparent. About one-half the tubes are removed from the water bath when the serum column will not flow if the tube is held in a horizontal position. This soft horse serum medium is then placed in the incubator for three days, and when found sterile the tubes are capped to prevent evaporation.<sup>1</sup> The remainder of the tubes

<sup>1</sup> The horse serum medium undergoes partial autolysis during this time and is preferable to horse serum not incubated.

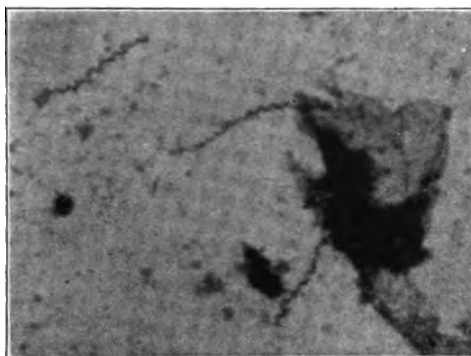


FIG. 5.—Cover glass smear from testicular lesion of a rabbit stained with Giemsa's method.

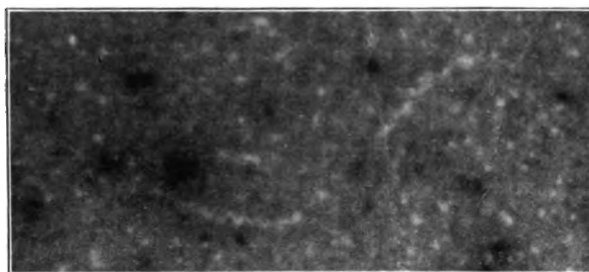


FIG. 6.—*Treponema pallidum* from a culture. An India ink preparation.

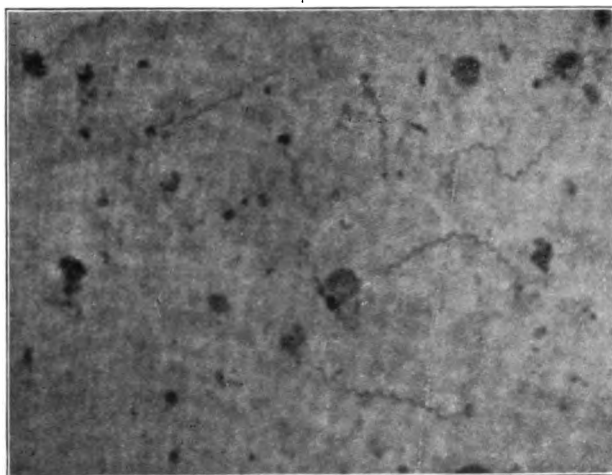


FIG. 7.—*Treponema pallidum* from a culture. Giemsa stain.

are allowed to reach a firmer consistency without, however, losing the transparency. This set of media is also tested for sterility, and then capped. The first lot of tubes are used for the initial step of cultivating the *treponema pallidum*. The growth of this organism is often hindered if too dense media are employed.

The horse serum used for the serum-agar is put into sterile test tubes, so that the tube is about one-third filled. The serum is then sterilized by placing it in a water bath for one hour at  $55^{\circ}\text{C}$ . for three successive days. A 3 per cent agar containing 0.5 per cent glucose is then melted and when cooled to  $50^{\circ}\text{C}$ . the tubes containing the heated serum are filled with melted agar equal to the amount of serum they already contain. It is advantageous to have sufficient assistance so that the tubes can be rolled to mix the agar and serum thoroughly. While the serum-agar in the test tubes is still liquid, the tubes are placed again in the water bath at  $55^{\circ}\text{C}$ ., which permits the media to clear. This requires about two hours. The medium will then be of a clear golden color and may be permitted to harden. The test tubes used are from 13 to 15 cm. in length. The serum-water plus tissue medium, as well as the ascitic-agar plus tissue medium used by Noguchi, is placed into tubes 20 cm. high. Such tubes are too long for the ordinary desiccators in laboratory use. To overcome this difficulty I have made use of large graduates with glass stoppers, with the mouth large enough to permit the passage of a large test tube. A rubber stopper with two holes is used instead of the glass stopper, and two glass stop-cocks are passed through the holes of the rubber stopper. The pyrogalllic acid is first placed in the graduate, then the inoculated tubes, and after that the rubber stopper with one of the glass cocks in place. To this glass cock is attached a rubber tubing long enough to reach to the bottom of the graduate. The second glass cock is then pushed through the rubber stopper. This causes the stopper to fit tightly. The same process is followed when desiccators are used for the cultivation of the *treponema pallidum* in coagulated horse serum and serum-agar media.

Although Noguchi found it necessary to begin the cultivation of the *treponema pallidum* in heated horse serum plus tissue, the

organism can be grown in coagulated horse serum, either by inserting a small piece of tissue containing the organisms, or by grinding up the tissue, diluting it with broth, and making inoculations into this medium by means of a capillary pipette. To inoculate with tissue it is best to use it in as fresh condition as possible. The tissue is divided into small pieces, the size of a split pea. With a sterile platinum wire the tissue is placed deep in the medium between the wall of the tube and the medium. The introduction of air bubbles must be avoided. The consistency of the media permits this method of inoculation without tearing. It has not been found necessary to place neutral oil on the surface of the media. The inoculated tubes are placed in a desiccator containing pyrogallic acid, and the desiccator is then exhausted and hydrogen passed through it until a test tube held to the outlet tube contains hydrogen. Suction is again applied to produce a vacuum in the desiccator and a strong solution of potassium hydroxid is run in through the outlet tube.

The alkaline pyrogallol solution is used in such proportion that one volume of a 22 per cent aqueous solution of pyrogallol is mixed with five or six times as much potassium hydroxid solution (3:2). At a temperature of 15° C. or higher the oxygen is quickly absorbed and a pyrogallol solution of the above concentration will not evolve carbon monoxid during the absorption.\*

Some investigators use rubber stoppers and dispense with the hydrogen, pyrogallic acid, and potassium hydroxid. The method employed by myself has seemed preferable because the surface of the cultures will be found entirely sterile, since the cotton plugs can be flamed and on subsequent examination of the cultures one can obtain the media containing the spirochaetes near the tissue, or liquid inoculated by means of a sterile pipette. The cultures should be examined about one week after inoculation, when one will find numerous characteristically motile spirochaetes if the inoculation has been successful. The work requires perseverance, and not all strains of the *treponema pallidum* will lend themselves to cultivation.

The pipette used is about 25 cm. long, drawn out, the upper end

\* Treadwell and Hall, *Analytical Chemistry*, Vol. 2, p. 686.

of such diameter that the rubber nipple will fit snugly. The lower end of the pipette is sealed and a hole is blown into it on the side, close to the lower end. This arrangement will prevent the media from rising into the lumen of the pipette when inoculations are made, or when it is desired to remove some of the culture for examination (Fig. 8).

The growing *treponema pallidum* may be observed to radiate from the tissue implanted in the coagulated horse serum in the form of a very faint cloud. The colonies are not sharply defined, nor distinct in their outline. The object of implanting the tissue near the wall of the test tube is to permit the growing *treponema* to penetrate into the medium. Thus one is enabled to obtain pure

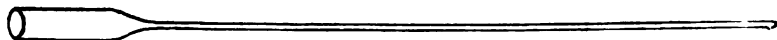


FIG. 8.—Pipette used for inoculation.

cultures. The cultures growing in the ascitic-agar plus tissue grow deeply at first and gradually the growth extends in a fine haze to within an inch of the surface of the medium. The coloring of the ascitic-agar by the hemoglobin from the tissue makes it very difficult to detect the colonies, and we have had tubes which, although appearing on inspection to be negative, were found to contain the organism when allowed to stand in the incubator for several weeks longer. Hazy colonies could then be detected above the reddened portion of the media. In the comparatively short time since this work was undertaken I have succeeded in growing one strain pure for four generations, and this strain was grown both in serum-water plus tissue and oil; ascitic-agar plus tissue and oil in a hydrogen atmosphere as well as in horse serum media, as described above and used by Mühlens, Hoffmann, and others. I have also succeeded in obtaining a typical syphilitic lesion in the testis of one of the two rabbits inoculated. The lesion was a circumscribed orchitis, not broken through the skin. This lesion, which was noticed five weeks after inoculation, was about the size of a cherry stone, and showed numerous actively motile spirochaetes

under the dark field. This culture was devoid of any unpleasant odor, whether grown in coagulated horse serum, serum-agar, serum-water plus tissue, or ascitic-agar plus tissue. Cultivation experiments with the other strains are in progress.

In conclusion it may be pointed out that the *treponema pallidum* of Schaudinn may be grown on various media and under varying conditions of anaerobiosis.

PRELIMINARY REPORT OF EXPERIMENTS IN THE  
VITAL STAINING OF TUBERCLES.\*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY  
OF TUBERCULOSIS. IV.

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of Chicago.)

In the rapid advance in our knowledge of scientific chemotherapy during the last few years, the various benzidine dyes have held no small or unimportant place. Since Ehrlich and Shiga<sup>1</sup> in 1904 published the results of their investigations on the trypanocidal action of trypan red, a new impetus has been given to the study of vital stains and especially to their use as therapeutic agents.

In 1906 Nicolle and Mesnil<sup>2</sup> reported an exhaustive and systematic study of dyes of the benzidine series (especially those resembling or closely related in their structure to trypan red) with regard to their vital staining and their trypanocidal power, the two being, they think, closely related. In the same year, Bouffard<sup>3</sup> published a communication along the same line, and numerous reports have come from Ehrlich and his workers, each adding something to our knowledge of vital stains which can be used therapeutically.

Goldmann<sup>4</sup> reported in 1909 an exhaustive investigation of the effect on the tissues of the normal body of a group of dyes, including the trypan red and the trypan blue already mentioned by Ehrlich and his workers, and comparing with them two other dyes which act in a similar way—isamine blue and pyrrhol blue. All these dyes have the common property of vitally staining granules in certain interstitial cells very deeply and permanently and thus giving a general and lasting stain to the body. After Goldmann's introduction of these dyes, a considerable number of workers

\*Received for publication, November 29, 1912.

<sup>1</sup> *Berl. klin. Wchschr.*, 1904, 41, p. 329; *ibid.*, 1907, 44, pp. 233, 280, 310, 341.

<sup>2</sup> *Ann. de l'Inst. Past.*, 1906, 20, p. 417.

<sup>3</sup> *Ibid.*, p. 539.

<sup>4</sup> *Beitr. z. klin. Chir.*, 1909, 64, p. 192.

investigated by means of these stains various physiologic and pathologic processes in the animal body, and during 1912 several communications from Goldmann have appeared, as well as two from Schulemann,<sup>1</sup> one from Lewis,<sup>2</sup> one from Bowman, Winternitz, and Evans,<sup>3</sup> and one from MacCurdy and Evans as well as several others which do not so especially have to do with my own work and which may therefore remain unmentioned.

Among all these workers, it is natural that some should be interested in the relation of these dyes to tuberculosis. Goldmann<sup>4</sup> himself in several of his later papers reports the results of his investigations on tuberculosis in mice, especially in regard to the origin and histogenesis of the tubercle. Bowman, Winternitz, and Evans, using rabbits, endeavored by means of the trypan blue dye to ascertain the histogenesis of the liver tubercle. Lewis showed that trypan red and isamine blue injected, intravenously or intraperitoneally, into tuberculous rabbits penetrated the pulmonary tubercles in the few cases which he reports.

Beyond suggesting the possibility that these results may some time be of use in the treatment of tuberculosis, none of these workers has taken up the chemotherapeutic value of vital stains in tuberculosis. Very recently, however, a series of papers has appeared dealing with the so-called "Finkler's Heilverfahren," the several papers being by Gräfin v. Linden,<sup>5</sup> Meissen,<sup>6</sup> Strauss,<sup>7</sup> and Selter.<sup>8</sup> The method uses either methylene blue (chlorid or iodid), or copper compounds, or both together. More or less favorable results were reported in the few experimental animals tested with the methylene blue treatment and also in a number of human tuberculous patients.

My own work began in February, 1912, and has been carried on with the definite purpose of testing dyes which can be used *intra vitam* to find if any have a specific action on the tubercle. The work is by no means finished, but certain results already obtained seem worth reporting at this time.

<sup>1</sup> *Berl. klin. Wchnschr.*, 1912, 49, p. 497; *Beitr. z. Vitalfärbung, Arch. f. mik. Anat.*, 1912, 79, p. 223; *Ztschr. f. exp. Pathol.*, 1912, 11, p. 307.

<sup>2</sup> *Arch. of Int. Med.*, 1912, 10, p. 68. <sup>3</sup> *Centralbl. f. Bakt.*, I, Orig., 1912, 65, p. 403.

<sup>4</sup> *Verhandl. d. path. Gesellsch.*, 1910, 14, p. 138; *Beitr. z. klin. Chir.*, 1912, 78, p. 1; *Lancet*, 1912, 182, p. 1183; *Berl. klin. Wchnschr.*, 1912, 49, p. 1689.

<sup>5</sup> *Beitr. z. Klin. der Tuber.*, 1912, 23, p. 201; *München. med. Wchnschr.*, 1912, 59, p. 2560.

<sup>6</sup> *Ibid.*, p. 215.

<sup>7</sup> *Ibid.*, p. 223.

<sup>8</sup> *Ibid.*, p. 261.



TABLE 1.

Name of Dye	Chemical Group	Chemical Structure
Trypan red.....	An azo dye	
Trypan blue.....	An azo dye	
Bismarck brown.....	An azo dye	
Methylene blue.....	Quinone imide Thiazin	
Neutral red.....	Quinone imide Eurhodine	
Janus green.....	Quinone imide Saffranin	
Brilliant cresyl blue.....	Quinone imide An oxazin dye	Structure not found
New methylene blue N..	Quinone imide A thiazin	
New methylene blue GG	Quinone imide An oxazin	
New methylene blue NX	Mixture of methylene blue 2B and methylene violet	Definite structure not found

TABLE 1—Continued.

Name of Dye	Chemical Group	Chemical Structure
New methylene blue R...	Chemical formula not found	
New methylene blue 3R	Chemical formula not found	
New methylene blue GB	Chemical formula not found	
New methylene blue NSS*	.....	
Eosin.....	Phenyl methane	
Basic fuchsin.....	Phenyl methane	
Crystal violet.....	Phenyl methane	
Pyronin.....	Phenyl methane	
Pyrrhol blue.....	Phenyl methane	
Isamine blue.....	Phenyl methane Formula not found	Reactions very similar to pyrrhol blue

\* A personal communication from the Casella Color Company informs me that the structure of new methylene blue NSS is that given for new methylene blue N, while the latter is a zinc double salt of the same formula.

TABLE 2.

Name of Dye	Water	Absolute Alcohol	Ether	Chloroform	Olive Oil	10 per cent HCl	10 per cent NaOH	10 per cent $\text{NH}_4\text{OH}$
Trypan blue.....	Freely soluble	Nearly insoluble	Insoluble	Insoluble	Insoluble	Soluble. No color change	Soluble. Reddish purple	Soluble. No color change
Trypan red.....	Freely soluble	Nearly insoluble	Insoluble	Insoluble	Insoluble	Soluble. No color change	Soluble. No color change	Soluble. Color deeper red
Bismarck brown..	Freely soluble	Freely soluble	Slightly soluble	Insoluble	Slightly soluble	Soluble. No color change	Slightly soluble. Color yellow	Slightly soluble. Color yellow
Eosin A.....	Freely soluble	Freely soluble	Insoluble	Insoluble	Insoluble	Insoluble	Soluble. Color bluish	Soluble. Color yellowish red
Eosin blue.....	Freely soluble	Freely soluble	Insoluble	Insoluble	Insoluble	Insoluble	Soluble. No color change	Soluble. Color deep red
Erythrosin.....	Freely soluble	Freely soluble	Slightly soluble	Slightly soluble	Insoluble	Nearly insoluble	Soluble. Color purplish	Soluble. Color deep red
Pyrrhol blue.....	Freely soluble	Freely soluble	Slightly soluble	Slightly soluble	Slightly soluble	Slightly soluble. No color change	Insoluble	Soluble. No color change
Ismine blue.....	Freely soluble	Freely soluble	Slightly soluble	Insoluble	Insoluble	Slightly soluble. Color purplish	Slightly soluble. Color reddish	Soluble. No color change
Basic fuchsin.....	Freely soluble	Freely soluble	Insoluble	Slightly soluble	Very slightly soluble	Slightly soluble. Color yellowish	Insoluble	Slightly soluble
Crystal violet.....	Freely soluble	Freely soluble	Insoluble	Soluble	Soluble	Soluble. Color yellowish green	Insoluble	Slightly soluble. No color change
Ehrlich's rectified methylene blue	Freely soluble	Freely soluble	Insoluble	Slightly soluble	Insoluble	Soluble. No color change	Very slightly soluble. Color reddish	Soluble. No color change
Medicinally pure methylene blue	Freely soluble	Freely soluble	Insoluble	Insoluble	Insoluble	Soluble. No color change	Very slightly soluble. Color reddish	Soluble. No color change
Neutral red.....	Freely soluble	Freely soluble	Insoluble	Soluble	Insoluble	Soluble. Color bluish	Slightly soluble. Color yellow	Nearly insoluble. Color yellow
Janus green.....	Freely soluble	Freely soluble	Slightly soluble	Slightly soluble	Slightly soluble. Color pink	Soluble. Color bluish	Slightly soluble. Color reddish	Soluble. Color green
Brilliant cresyl blue.....	Freely soluble	Freely soluble	Insoluble	Slightly soluble	Insoluble	Soluble. Color reddish	Nearly insoluble	Soluble. Color reddish
Diamine schwarz.	Freely soluble	Freely soluble	Slightly soluble	Slightly soluble. Color reddish	Insoluble	Insoluble	Slightly soluble. Color reddish	Freely soluble. Color bluish
New methylene blue N.....	Freely soluble	Freely soluble	Insoluble	Slightly soluble	Insoluble	Soluble. No color change	Insoluble	Freely soluble. Color deep blue
New methylene blue GG.....	Freely soluble	Freely soluble	Slightly soluble. Color pink	Soluble. Color reddish purple	Soluble. Color red	Soluble. Color blue	Soluble. Greenish blue changing to brown	Soluble. Color greenish blue
New methylene blue NX.....	Freely soluble	Freely soluble	Slightly soluble	Freely soluble	Soluble. Color reddish	Soluble. Color green changing to blue	Slightly soluble. Color blue	Freely soluble. Color deep blue
New methylene blue R.....	Freely soluble	Freely soluble	Insoluble	Freely soluble	Soluble. Color bluish red	Soluble. Color green changing to blue	Insoluble	Slightly soluble. Color blue
New methylene blue 3R.....	Freely soluble	Freely soluble	Insoluble	Freely soluble	Soluble. Color bluish red	Soluble. Color green changing to blue	Insoluble	Slightly soluble. Color reddish
New methylene blue GB.....	Freely soluble	Freely soluble	Insoluble	Freely soluble	Soluble. Color bluish red	Soluble. Color green changing to blue	Slightly soluble. Color blue	Soluble. Color blue
New methylene blue NS.....	Freely soluble	Freely soluble	Insoluble	Freely soluble	Soluble. Color bluish red	Soluble. Color green changing to blue	Insoluble	Slightly soluble. Color pink
Pyronin.....	Freely soluble	Freely soluble	Insoluble	Slightly soluble	Insoluble	Soluble. No color change	Insoluble	Slightly soluble. Color pink

Having constantly a chemotherapeutic aim in mind, the following problems must be solved in the study of each dye:

1. Will the dye penetrate the tubercle? This being a non-vascular structure, the question of penetration itself presents no inconsiderable difficulties.

2. Is it toxic to the animal? In a slow, chronic process like tuberculosis, a remedial agent must be one which is well borne by the patient and can be administered at intervals for long periods, if necessary. We can scarcely hope, as may be possible in acute infections, like many of the trypanosomiasis, to complete the sterilizing and curative process by a single treatment.

3. Will the dye penetrate the tubercle bacillus? We can be sure of this only if the bacilli stain readily, clearly, and permanently.

4. If the dye penetrates the tubercle bacillus, does it kill it or injure its vitality or inhibit its growth?

5. If the dye does not itself kill the bacilli, can its structure be modified, can new side chains be introduced or can metals or other elements be substituted in its molecule to make it more bactericidal without at the same time interfering with its penetrating power and its innocuousness?

Table 1 gives a list of the dyes which have been used so far in the work with their chemical formulae as far as I have been able to find them.

The preparations of these dyes used by myself have been tested as to their solubilities in the ordinary reagents and as to their reactions to acids and alkalis. The results are given in Table 2. This is important since different preparations often differ in their solubilities and reactions.

I have tested the staining power of most of these dyes on fixed and unfixed tissues. Tissues were fixed in a chrome-formalin solution, imbedded in paraffin, and sectioned. The sections, after removal of paraffin, were stained with one per cent water solutions of the dyes, with the following results:

1. Trypan blue: diffuse light protoplasmic stain with much darker nuclei. No coarse blue granules.
2. Trypan red: diffuse protoplasmic stain, nuclei sometimes slightly darker. No granular stain.
3. Bismarck brown: sharp nuclear stain. Protoplasm light yellow.

4. Eosin A } diffuse protoplasmic stain.
5. Eosin B }
6. Erythrosin: diffuse protoplasmic stain. In some areas nuclei slightly darker.
7. Pyrrhol blue: nuclei darker than protoplasm.
8. Isamine blue: protoplasm diffusely stained. No differentiation of nuclei.
9. Basic fuchsin: nuclei differentiated.
10. Crystal violet: nuclei somewhat darker than protoplasm. Differentiation not very sharp.
11. Pyronin: slight nuclear stain.
12. Methylene blue (both rectified and medicinally pure): sharp nuclear differentiation.
13. Neutral red: sharp nuclear differentiation.
14. Janus green: sharp nuclear differentiation.
15. Brilliant cresyl blue: sharp nuclear stain.
16. New methylene blue N: very good, sharp nuclear stain in short time.
17. New methylene blue GG: good, sharp nuclear stain.

Fresh tissues from a guinea-pig were dropped while still warm in one per cent water solutions of the dyes, kept at room temperature, and removed at the end of 24 hours. Some portions were examined at once, while others were fixed, dehydrated, imbedded in paraffin, and sectioned. The latter method gave good results with a few of the dyes, but most of them were soluble in alcohol, so that the dye diffused through the tissue and no satisfactory results could be gained. Most of the results reported are, therefore, from the tissue immediately after removal from the stain. The results are as follows:

1. Trypan blue: no change in dye. Tissue deep blue. Sharp nuclear stain; no granular stain. Paraffin sections gave the same appearance.
2. Trypan red: no change in dye. Tissue deep red. Diffuse stain of protoplasm. No granular stain. In some areas nuclei were darker than protoplasm. Same appearance in the paraffin sections.
3. Erythrosin: diffuse protoplasmic stain. No nuclear differentiation. Much diffusion of stain in imbedding, but appearance in the main was the same in the sections.
4. Isamine blue: not stained at all.
5. Pyronin: protoplasm faintly stained; nuclei somewhat more deeply. Much diffusion of stain in imbedding.
6. Methylene blue: penetration not very good. Periphery well stained. Good nuclear differentiation; some diffusion of stain in imbedding, but nuclear stain was still quite sharp in sections.
7. Neutral red: penetration not very good. Sharp nuclear stain at periphery. In sections, stain more diffuse and differentiation of nuclei not so good.
8. Brilliant cresyl blue: penetration very poor. On surface, a good nuclear stain, still retained in the paraffin sections.

9. New methylene blue N: sharp nuclear stain at surface, but penetration was poor. Nuclear stain well preserved in sections.

10. New methylene blue GG: very little penetration. Nuclear stain at surface. Well preserved in sections.

Most of these dyes, as may be seen from these results, react to the nuclei as bases, either strongly or weakly, even though they have an acid chemical structure. A number of the dyes which have more recently been added to my list have not yet been subjected to the above tissue test.

In the animal experiments, one per cent water solutions of the dyes have been used in most of the experiments, although with the dyes which have been shown to be irritating in that concentration one-fourth of one per cent and even one-tenth of one per cent solutions have been employed. Most of the injections have been subcutaneous, but in some cases the dyes have been injected intraperitoneally. In all cases guinea-pigs which had been inoculated subcutaneously with human tuberculosis were used. The results of the animal experiments both as to the penetration of the tubercle and as to injury to the animal are briefly given in Tables 3 and 4. Since trypan blue has been more thoroughly tested than any of the other dyes, the work with this dye has been summarized by itself in Table 3, while Table 4 states briefly the work so far finished on the other dyes used.

Sixteen tuberculous guinea-pigs were injected for longer or shorter periods with one per cent aqueous solution of trypan blue, from 6 to 10 c.c. being given at each dose. This was always well borne and no local or general toxic or irritative effects were noticed in any of the animals, except that the pregnant pigs always aborted, and most of them died shortly after the abortion. This may be due to the concentration of the dye in the placenta, which was noted by Goldmann and which I also have observed, but it is not in agreement with his observation on mice, as he says that these pass normally through several pregnancies, with no ill effects from the dye. In nearly every case, the smaller tubercles of lungs, liver, and spleen have taken the stain and are usually sharply contrasted with the surrounding tissue, which is either unstained or less deeply stained than the tubercles. The larger necrotic areas

TABLE 3.  
ANIMAL EXPERIMENTS WITH TRYPAN BLUE.

Number	Method of Injection	Number of Injections	Concentration of Dye	Age of Infection	Autopsy Findings	Result of Dye Injections on Tubercle
1....	Subcutaneous	3	1 per cent	59 days	Severe local and general tuberculosis	Tubercles all blue. Central mass unstained
2....	Subcutaneous	5	1 per cent	90 days	Severe local and general tuberculosis	Tubercles all blue. Pus blue. No stained bacilli. No growth on agar tubes
3....	Subcutaneous	3	1 per cent	107 days	Severe local, no general tuberculosis	Tuberculous gland blue. Caseous center blue. No stained bacilli
4....	Subcutaneous	4	1 per cent	15 days	General miliary tuberculosis with peritonitis	Tubercles blue, but not deeper than surrounding tissue
5....	Subcutaneous	8	1 per cent	126 days	Severe local and general tuberculosis	All tubercles bright blue. Caseous and softened centers bright blue
6....	Subcutaneous	1	1 per cent	92 days	Severe local and general tuberculosis. Large necrotic spleen	All tubercles blue. Necrotic areas especially blue. Normal spleen unstained
7....	Subcutaneous	4	1 per cent	126 days	Severe local and general tuberculosis	All tubercles blue. Liver and spleen tubercles especially blue
8....	Subcutaneous	6	1 per cent	72 days	Local glandular and spleen tuberculosis	All tubercles blue. Caseous centers deep blue. No distinctly stained bacilli, but stained granules
9....	Subcutaneous	1	1 per cent	69 days	Severe local, no general tuberculosis	Periphery of tuberculous gland blue. Center not stained
10....	Subcutaneous	3	1 per cent	81 days	Severe local, no general tuberculosis	Tuberculous glands blue. Caseous centers blue. Bacilli not stained
11....	Subcutaneous	4	1 per cent	100 days	Severe local, slight general tuberculosis	All tubercles blue. Caseous centers blue. No stained bacilli found
12....	Subcutaneous	4	1 per cent	100 days	No local, no general tuberculosis	Distribution of dye as in normal pigs
13....	Subcutaneous	2	1 per cent	119 days	Severe local and general tuberculosis	All tubercles blue. Caseous centers blue. Necrotic areas very blue
14....	Intraperitoneal	1	1 per cent	90 days	Severe local and general tuberculosis	All tubercles blue
15....	Intraperitoneal	1	1 per cent	93 days	Severe local and general tuberculosis	All tubercles blue
16....	Intraperitoneal	1	1 per cent	93 days	Severe local and general tuberculosis	All tubercles very blue. Very little stain in normal tissue

so frequently present in the liver and spleen of the tuberculous guinea-pig take the stain with especial avidity. The large caseous and softened lymph glands always show a blue periphery, and if the injections have been repeated a number of times (*hochgetrieben*, as Goldmann calls it) the caseous and softened centers are also deep blue, and often deep blue pus exudes from the tubercle for some weeks before death. Distinctly blue stained tubercle bacilli have never been noted, either in this blue pus or in the smaller tubercles, although at times deeply blue stained granules are seen which

appear to be in bacilli, and these may retain a blue stain to some extent even after the bacilli have been stained with carbol fuchsin. The staining of tubercle bacilli in the body with trypan blue has not, however, been satisfactorily proven. Cultures from the blue stained pus on glycerin agar have been made several times, but no growth has developed, and animal inoculations from this pus have not been made.

On microscopic examination, the smaller tubercles in the trypan blue sections show a peripheral zone of cells filled with coarse deep blue granules, cells like those found everywhere in the interstitial tissue and which are probably the cells described by Goldmann as *Pyrrholzellen*, although the granules are somewhat larger and more irregular in size than in the normal cells.

Central to this is a zone which shows very little blue stain, but which, in sections stained with sudan III, exhibits many red-stained droplets and granules (fat). The center of the tubercle is blue, more or less diffuse or filled with fine granules with a few coarser ones intermingled. This appearance is so different from any seen in the normal tissues stained with trypan blue that such an area can readily be picked out under a very low magnification. The color is bright blue as contrasted with the dark blue or blue black of the intracellular granules normally dyed by this stain when injected into the living animal. Tuberculous giant cells, when present, show a diffuse pale blue center with somewhat darker blue nuclei around the periphery. Goldmann describes similar findings in his mice inoculated with avian and also with bovine tuberculosis. He states that through the bacterial invasion and through the influence of the bacilli, the cells suffer an injury which expresses itself in the gradual loss of granular structure. The vital dye, anchored by the granules, after solution of the granules, first appears in coarser granules and later diffuse in the cell protoplasm and then finally disappears. The cell injury, he states, is only partial, since the nuclei remain unchanged and stainable and showing no degenerative phenomena even after perfect disappearance of the granules. He thinks that with the loss of granular structure, the cells lose their ameboid motility, and other cells collect around them forming larger and smaller cell conglomerates in which all transition stages



TABLE 4.  
ANIMAL EXPERIMENTS WITH DYES.

Number	Dye Used	Concentration	Method of Injection	Number of Injections	Time of Action of Dye	Age of Infection	Effect of Dye during Life	Autopsy Findings	Postmortem Dye Findings
1.....	Janus green....	1 in 1,500	Into aorta after bleeding	1	None	54 days	None	Local and general tuberculosis	Tubercles unstained. Normal tissues well stained
2.....	Janus green	$\frac{1}{4}$ per cent	Subcutaneous	4	8 days	3 months	Infiltration and sloughing	Local, no general tuberculosis	Tubercle unstained. Other tissues little stained
3.....	Pyronin.....	$\frac{1}{4}$ per cent	Subcutaneous	3	6 days	?	No ill effects	Severe local and general tuberculosis	Tubercles unstained. Other tissues unstained
4.....	Neutral red	$\frac{1}{4}$ per cent and 1 per cent	Subcutaneous	6	16 days	?	No ill effects	Local, no general tuberculosis	Tubercles very slightly stained
5.....	Neutral red	1 per cent	Subcutaneous	4	56 days	65 days	No ill effects	Local and general tuberculosis	Tubercles in lung very faintly stained
6.....	Neutral red	1 per cent	Subcutaneous	7	73 days	107 days	No ill effects	Local, no general tuberculosis	Tubercles unstained
7.....	Neutral red	1 per cent	Subcutaneous	10	25 days	?	No ill effects	Local, no general tuberculosis	Periphery of caseous glands stained. Center unstained
8.....	Erythrosin	1 per cent	Intra-aortic	1	None	71 days	None	Severe local and general tuberculosis	Tubercles and necrotic areas unstained. Other tissues bright red
9.....	Rectified methylene blue	1 in 2,000	Intra-aortic	1	None	60 days	None	Local and general tuberculosis	Tubercles unstained. Other tissues well stained
10.....	Rectified methylene blue	$\frac{1}{4}$ per cent	Intra-aortic	1	None	46 days	None	Local and general tuberculosis	Tubercles unstained. Other tissues stained
11.....	Rectified methylene blue	$\frac{1}{4}$ per cent	Subcutaneous	4	7 days	?	Infiltration and sloughing	Slight local, severe general tuberculosis	Tubercles unstained. Other tissues unstained
12.....	Rectified methylene blue	$\frac{1}{4}$ per cent and 1 per cent	Subcutaneous	6	14 days	?	Infiltration and sloughing	Local and general tuberculosis	Tubercles well stained after oxidation of dye
13, 14, 15, 16.....	Medicinally pure methylene blue	1 per cent	Subcutaneous	2	5 to 8 days	?	Much infiltration	No tubercles found	Tissues blue
17.....	Medicinally pure methylene blue	$\frac{1}{4}$ per cent	Subcutaneous	4	7 days	?	Infiltration and sloughing	Local and general tuberculosis	Tubercles very little stained

18.....	Medicinally pure methylene blue	‡ per cent	Subcutaneous	1	2 days	?	Some infiltration	Local, no general tuberculosis	No stain anywhere
19.....	New methylene blue N	‡ per cent and 1 per cent	Subcutaneous	9	28 days	?	Slight infiltration. Well borne	Local and general tuberculosis	Internal tubercles blue after oxidation. Bacilli not stained
20.....	New methylene blue GG	‡ per cent and 1 per cent	Subcutaneous	7	23 days	?	Slight infiltration. In main well borne	Local and general tuberculosis	All tubercles blue after oxidation. No stained bacilli
21.....	Methylene blue of U.S. Pharmacopoeia	1 per cent	Subcutaneous	4	8 days	?	Marked infiltration and sloughing	Local and general tuberculosis	Internal tubercles stained. No stained bacilli
22.....	Same as 21	1 per cent	Subcutaneous	1	1 day	?	No ill effects	Local and general tuberculosis	Internal tubercles deep blue after oxidation. No stained bacilli
23.....	Trypan red	1 per cent	Subcutaneous	7	7 days	?	No ill effects	Local and general tuberculosis	All tubercles stained deep red; also pus of caseous glands
24.....	Trypan red	1 per cent	Intraperitoneal	1	6 days	82 days	No ill effects	Local and general tuberculosis	All tubercles stained. No stained bacilli
25.....	Trypan red	1 per cent	Intraperitoneal	1	10 days	92 days	No ill effects	Local and general tuberculosis	All tubercles stained. No stained bacilli
26.....	Trypan red	1 per cent	Intraperitoneal	1	7 days	87 days	No ill effects	General glandular tuberculosis. Liver, spleen, and lungs not involved	Periphery of all tuberculous glands red. Centers unstained
27.....	Bismarck brown	1 per cent	Subcutaneous	3	6 days	?	None	No tuberculosis	No stain anywhere
28.....	Bismarck brown	1 per cent	Subcutaneous	3	7 days	?	No ill effects	No tuberculosis	No dye anywhere

may be seen. He also notes that with the change in the granular structure of the cell, and especially after the granules have entirely disappeared, a fat reaction is given by appropriate dyes.

The large necrotic areas of liver and spleen also have the bright blue diffuse appearance seen in the true tubercle. These areas take up the stain more quickly and readily even than the true tubercles.

The manner in which the normal tissues react to trypan blue when introduced during life has been so well described in the various communications by Goldmann and others that no detailed description is needed here. It is sufficient to say that in nearly all organs and in the skin, he found certain cells of the interstitial tissue which take up the stain in granular form, these granules being rendered visible by the dye, but being invisible when the dye has not been used. He calls these cells *Pyrrholzellen*. It is impossible to avoid the thought that this stain in normal tissues may be due to a phagocytic action, especially as the cells most active in taking up the dye are active phagocytes toward other foreign substances. Goldmann, however, is very sure, from his observations, that the action is not phagocytosis, and Gross asserts that it is due to a special activity of the cells, while Schulemann thinks that the granules are *Reaktionskörper* formed by the entrance of the dye into the cells. Without discussing this question further at this time, it may be said that the staining of the tubercle and of the necrotic tissue is quite different from that of the normal tissue, responding to quite a different law. While the nuclei of normal living cells probably never stain with trypan blue, the nuclei of the tuberculous giant cells are especially stained; also the diffuse, hyaline appearing stain of the protoplasm is probably rarely, if ever, found in the normal tissue vitally stained by trypan blue. It may be said, however, that this stain is not specific for the tubercle. Numerous investigators have demonstrated that injured kidney cells show nuclear stain and diffuse protoplasmic stain with trypan blue. Goldmann states that in experimentally produced liver necrosis a similar change in the normal reaction to the dye occurs. Brown and Evans observed a similar stain of cells of the central nervous system in experimental anterior poliomyelitis, and in several guinea-pigs I caused necrosis

of muscle by injection of formalin and found the muscle, which normally takes up no or very little trypan blue, staining deeply and diffusely blue. The same phenomenon was noted by Ribbert<sup>1</sup> as to the action of carmine granules introduced during life; the usual granular appearance being lost and the injured cells showing diffusely stained protoplasm with darker nuclei.

Even though not specific for the tubercle, however, an important point is gained in that we have in trypan blue a dye which penetrates the tubercle quite easily, and remains in it for a considerable period. I have found tubercles well stained in animals which were killed as late as nine days after the last injection, while I have also found them well stained in animals killed or dying as early as 12 hours after a single injection. We have, then, in trypan blue a dye which penetrates the tubercle easily and quickly and remains for some time fixed in the tubercle. It is also practically innocuous for the animal, as I have in many cases given 8 to 10 c.c. of one per cent solution several times a week for weeks with no noticeable ill effect, either to the general condition or locally at the point of injection. It is one of the dyes, which, like trypan red, was found by Ehrlich and his workers to have a marked trypanocidal power over some kinds of trypanosomes. It is therefore worthy of careful study with relation to its bactericidal action in tuberculosis. The results of my work in this direction will be given more in detail a little later in this report. It may be said that the animals listed in Table 3 received no injections of the dye until very late in the disease, and very little attention was at this time paid to curative properties, most of the animals being killed, without waiting to see how long they might have lived under the treatment. My whole attention at this stage of the work was given to the penetrating and staining power of the dye.

Table 4 gives a series of experiments with various dyes as to their penetrating power and their toxicity for the animal. From this table, although in general the number of experiments with each dye is small, the following facts are noted:

1. Janus green is unpromising for treatment because it causes deep sloughing if used subcutaneously for any time and does not penetrate the tubercle.

<sup>1</sup> *Ztschr. f. allg. Physiol.*, 1904, 4, p. 201.

2. Pyronin: the dye is well borne but rapidly excreted and seems not to penetrate the tubercle. Or, if it does, it passes out of it quickly, since the pig was killed within 12 hours after an injection and no stain, or very little, was found in the tubercle.

3. Neutral red: this dye is well borne by the animal but shows very little, if any, power to penetrate the tubercle. Further experiments will be made with this dye.

4. Erythrosin: only one experiment and that a post-vital, intracardiac injection. In this the stain did not penetrate the tubercles. As the dye seems to be quite well borne and is in other respects favorable, further experiments and a later report will be made on this dye.

5. Methylene blue: several preparations have been used:

a) Ehrlich's rectified methylene blue, which in post-vital, intracardiac injection did not penetrate the tubercle. In subcutaneous, repeated injections, it penetrated the smaller tubercles of lungs, liver, and spleen, but not to the softened centers of caseous glands. In the small tubercles, the dye was reduced to the leukobase, so that no color appeared until after exposure to air and sometimes not until after the tissue was placed in ammonium molybdate solution. Then the tubercles became intensely blue and remained so during the process of dehydrating and imbedding in paraffin.

In a one-fourth of one per cent solution, injected subcutaneously, it caused much induration and some sloughing, but in weaker solutions and in small amounts it is well borne.

b) Methylene blue—medicinally pure: the results of the experiments finished have been about the same as with Ehrlich's rectified methylene blue.

c) Methylene blue of the U.S. Pharmacopeia: results were the same as with other methylene blues, but local reaction was not so severe.

A larger number of tuberculous guinea-pigs are now being systematically treated with this dye, a report of which will be published later.

d) New methylene blue N.

e) New methylene blue GG.

Both (d) and (e) give the same general results as the ordinary methylene blues and are about as well borne by the animals.

Further experiments with these dyes are in progress as well as with other new methylene blues.

6. Trypan red: this is the dye first shown by Ehrlich and Shiga to have the power to destroy trypanosomes in the animal body. My experiments, while not so numerous as those with trypan blue, show that the dye readily penetrates the tubercle, although not quite so readily as does trypan blue. No ill effects have been observed in guinea-pigs from the use of this dye. My results with this dye have practically agreed with the experiment reported by Lewis on the rabbit.

7. Bismarck brown, while apparently innocuous to the animal, is rapidly excreted, and I could find no trace of the dye in any of the organs twelve hours after the last injection. The guinea-pigs used proved non-tuberculous, so that the penetration of the tubercle was not tested.

Other dyes are now being tested, and report of their influence will be published later. Other dyes and dye modifications are being worked out. So far, then, we find that trypan blue, trypan red, to a slight extent neutral red and pyronin, isamine blue, as tested

by Lewis, and pyrrhol blue, which acts in all respects like isamine blue, Ehrlich's rectified methylene blue, Pharmacopeial methylene blue, medicinally pure methylene blue, new methylene blue N, and new methylene blue GG may penetrate the tubercles with less or greater ease and are borne for long periods in larger or smaller doses with no great inconvenience.

The next question of importance then is: Do these dyes penetrate the tubercle bacillus? Do they stain the dead, fixed bacillus? Do they stain the living bacillus *in vitro*? Do they stain the bacillus in the tubercle? Numerous tests have been made with the various dyes to determine their power to stain the tubercle bacilli. It may be stated that human tubercle bacilli grown on glycerin agar have been used almost exclusively. The following methods have been tested for staining: (1) inoculating with tubercle culture tubes of glycerin agar to which had been added varying quantities of the dyes; (2) inoculating glycerin agar tubes with tubercle bacillus cultures, and, after these were somewhat grown, filling up the tube with one per cent water solutions of the dyes; (3) adding a small amount of suspension of tubercle bacilli to the dye, and, after certain intervals of time, washing, centrifuging, and making smears; (4) ordinary smears on slides, fixed by heat, were placed in one per cent water solutions of the dyes, sometimes hot and sometimes cold, and examined at the end of different periods of time. The final results in most cases were obtained at the end of 20 to 24 hours at 39° C. temperature. This of course does not apply to the growth experiments on stained glycerin agar, as in these the macroscopic staining was noted at the end of 24 hours, but the microscopic appearance only at the end of a growth period of four to six weeks. As the results of all these tests corresponded in a general way, it will be sufficient to state the general results without reporting the various experiments in detail.

1. Trypan blue gives a good mass stain, but individual bacilli are but faintly stained, and most of the individuals not at all. As this dye had proved so promising for use in animal experiments, very special efforts were made to strengthen its staining power and to obtain a good individual stain of the bacilli. The following methods were used:

a) Heat-fixed smears of tubercle bacilli were stained 24 hours in cold one per cent water solution of trypan blue. Groups of bacilli and some few individuals were faintly stained.

b) Heat-fixed smears of tubercle bacilli remained 24-48 hours in one per cent water solution of trypan blue at 39° C. Stain was slightly deeper both in groups and in individuals, and granules were stained dark in some bacilli. Most of the individuals, however, remained unstained.

c) Smears were stained as in (b) and then washed in 20 per cent formalin. A heavy precipitate formed, but the stain was not improved.

d) Smears were fixed in absolute methyl alcohol instead of by heat and staining attempted by various methods, but results were no better than in heat-fixed smears.

e) Potassium hydroxid was applied to the smear before staining, but the results were no better.

f) An agar slant culture of human tubercle bacilli was covered with one per cent water solution of trypan blue and left in the incubator for 24 hours. The dye was then poured off, the slant washed, and a portion of the culture spread on slides. The stain seemed somewhat clearer and darker, but still not very clear. Not all bacilli were stained and even the best stained still appeared somewhat shadowy.

g) Five per cent of phenol was added to the dye and this was used on the agar slant as in (f) and the tubes were left in the incubator 24-48 hours. The stain appeared darker and more granules were seen.

h) Formalin was used in the same way as phenol under (g) and with about the same results.

i) Potassium hydroxid was used in same way as phenol in (f) but the stain was not at all improved.

j) All the above methods were used on strong thick emulsions of tubercle bacilli, afterward washing and centrifuging. The results were not materially different from those obtained from agar slants. From all these tests, it may be stated that trypan blue readily stains a mass of bacilli and perhaps also the outer covering of some individuals, but does not readily or satisfactorily penetrate individual bacilli.

2. Trypan red also gives a fairly good mass stain, but the individual stain is even fainter and more unsatisfactory than that of trypan blue.

3. Methylene blue: five modifications of this dye have been tested as to their power to stain tubercle bacilli—Ehrlich's rectified methylene blue, medicinally pure methylene blue, the methylene blue of the U.S. Pharmacopeia, and the new methylene blue N and new methylene blue GG. All these preparations stain masses of tubercle bacilli quickly and intensely—all readily stain individual bacilli clearly, deeply, and permanently. This latter action is more intense on the unfixed bacilli of the emulsion or agar slant than on the smear fixed by heat. But even in the latter there is no question that the individual bacilli are well and permanently stained. Granules within the bacilli are often more intensely stained than the rest of the organism.

4. Eosin A, eosin blue, and erythrosin all stain both masses of bacilli and single bacilli clearly and permanently. These three dyes were used always in one per cent water solution, and, while they stained the smears fixed by heat very well, they all acted most intensely on the fresh, living, or unfixed organism. Erythrosin, of the three preparations, stained the bacilli most intensely, and eosin A least.

5. Neutral red gave a good mass stain, but a very faint individual stain.

6. Pyronin also gave good mass stain but very faint individual stain, or none at all.

7. Janus green also gave a fair mass stain but practically no stain of individual tubercle bacilli.

8. Gentian violet stains quickly and intensely both masses of bacilli and single organisms.

9. Basic fuchsin also stains very deeply both masses of tubercle bacilli and individuals.

10. Brilliant cresyl blue has very little power to stain either masses of tubercle bacilli or single organisms.

11. Bismarck brown stains masses of bacilli very well, but usually dyes individual organisms but faintly. Occasionally I have had preparations in which the stain was clearer and deeper than usual.

12. Isamine blue and pyrrhol blue are much like trypan blue in their good mass staining with faint, weak individual staining.

So it may be seen that practically all the dyes studied stain masses of tubercle bacilli well in a very few minutes, but it is much more difficult to penetrate the individual organism and color its interior. Most of the dyes seem simply to form some kind of combination with the bacillary membrane and not to be able to pass through it. Only gentian violet, basic fuchsin, erythrosin, and the eosins, the different methylene blue preparations, and the new methylene blues may be said to stain uniformly and satisfactorily the interior of the tubercle bacilli, and this, in the question of the therapeutic value of a substance, is of prime importance.

In connection with the penetration and staining of tubercle bacilli by these various dyes, the question of their bactericidal power is of very great importance. The dyes used throughout these experiments were first tested for their bactericidal or their inhibiting action *in vitro* on a number of ordinary organisms. Several methods were used to test the bactericidal or inhibiting action of the dyes on the various organisms. The bacteria used have been *B. coli*, *B. typhosus*, *B. dysenteriae* (Shiga), *B. prodigiosus*, *B. pyocyaneus*, *B. subtilis*, *Staphylococcus aureus*, and an acid-fast organism supposed to be an old attenuated human tubercle culture. Often the growth was reduced in amount and the colonies in number, even though growth took place. The methods used have been: (1) a somewhat modified Anderson-McClintock method, in which cultures were made both in broth and on agar slants, the latter bringing out the quantitative relation much better than the broth; (2) adding definite amounts of dye to agar tubes and then



inoculating and watching them; (3) broth cultures of the different organisms were grown at room temperature for 72 hours; then 10 drops of one per cent water solution of the dye were added to each tube containing 10 c.c. of a moderately thick bacterial emulsion; after certain definite times, a loopful from each tube was streaked on agar slants. Table 5 gives briefly the results of one of the experiments, and, as all the results agreed fairly well, this may stand as the report of this work.

TABLE 5.

GROWTH EXPERIMENTS OF ORDINARY BACTERIA ON STAINED AGAR. TWO DROPS OF ONE PER CENT SOLUTION OF DYE TO EACH TUBE OF AGAR

Dyes Used	<i>B. tuberculosis</i> *	<i>Staphylococcus aureus</i>	<i>B. subtilis</i>	<i>B. typhosus</i>	<i>B. pyocyaneus</i>	<i>B. dysenteriae</i> (Shiga)	<i>B. prodigiosus</i>	<i>B. coli</i>
Basic fuchsin....	—	+	—	+++	+++	+	+++	+++
Bismarck brown....	—	+++	+++	+	++	+	+++	+++
Trypan red.....	+++	+++	+++	+++	+++	+	+++	+++
Trypan blue.....	+++	+++	+++	+++	+++	+	+++	+++
Isamine blue.....	+++	+++	+++	+++	+++	+	+++	+
Ehrlich rectified methylene blue	—	—	—	+	+++	+	+	+
Medicinally pure methylene blue	—	—	—	+	+++	+	+	+
New methylene blue N.....	—	—	—	+++	+++	+	+	+++
New methylene blue GG.....	—	—	—	+++	+++	+	+	+
Eosin A.....	+++	+++	+++	+++	+++	+	+++	+++
Eosin blue.....	+++	+++	+++	+++	+++	+	+++	+++
Erythrosin.....	+	+++	+++	+	+++	+	+++	+++
Pyronin.....	+	—	—	+++	+++	+	+++	+++
Janus green.....	+	—	—	+++	+++	+	+++	+
Neutral red.....	+	—	+++	+++	+++	+	+++	+++
Gentian violet.....	+	—	—	+++	+++	+	+++	+++
Pyrrhol blue.....	+++	+++	+++	+++	+++	+	+++	+++
Brilliant cresyl blue.....	+	—	—	+++	+++	+	+	+
Control.....	+++	+++	+++	+++	+++	++	+++	+++

\* The organism in column one was an acid-fast organism, supposed to be an old, attenuated tubercle bacillus culture, but its history was not exactly known. It grows rapidly and luxuriantly, and produces abscesses in guinea-pigs. These abscesses are acute or at least develop much more rapidly than tuberculous glands, and other pigs in the same cage are infected.

— Signifies no growth.

+ Signifies little growth.

++ Signifies moderate growth.

+++ Signifies luxuriant growth.

From this table it may be seen that the questionable tubercle bacillus was killed or inhibited by basic fuchsin, bismarck brown, Ehrlich's rectified methylene blue, medicinally pure methylene blue, new methylene blue N, new methylene blue GG, and Janus green, and its growth was diminished by erythrosin, eosin, pyronin, gen-

tian violet, brilliant cresyl blue, and neutral red. *Staphylococcus aureus* showed no growth in the tubes containing any of the methylene blues, pyronin, Janus green, gentian violet, brilliant cresyl blue, and neutral red. *B. subtilis* failed to develop in tubes containing the five methylene blues, pyronin, Janus green, gentian violet, and eosin blue. While other organisms were much diminished in their growth by certain dyes, none of them showed an entire absence of growth. While these results do not agree in all details with those obtained by Churchman in his work on the bactericidal action of gentian violet, they verify his statement that gentian violet and also other dyes not mentioned by him have a certain selective bactericidal action. It should be noted here that this action noted in Table 5 is partly inhibitory, especially so far as the methylene blues are concerned. In the experiment in which 10 drops of the dyes were added to thick broth cultures, none of the cultures were killed by the methylene blue even after 24 hours' exposure, although the growth was diminished in some cases. In this experiment, no growth of the acid-fast organism developed after exposure 24 hours to bismarck brown, erythrosin, pyronin, Janus green, eosin, while the basic fuchsin, trypan blue, and gentian violet tubes gave from one to three colonies. *Staphylococcus aureus* failed to develop after 24 hours' exposure to basic fuchsin, pyronin, Janus green, and eosin. *B. subtilis* showed some growth on all the tubes, but only three to five colonies in the tubes after exposure 24 hours to erythrosin, pyronin, Janus green, eosin, and gentian violet. The slight differences in these results are perhaps due to the fact that in the broth culture after three days' growth, many larger clumps had formed which were not easily penetrated by the methylene blues and some of the other dyes.

An interesting fact was noted in these experiments—that all the methylene blues and brilliant cresyl blue are reduced by the growth of certain organisms, especially in this experiment, by *B. typhosus*, *B. coli*, *B. prodigiosus*, and *B. pyocyaneus*. In all these tubes the color was quickly restored by the addition of hydrogen peroxid.

The bactericidal action of these dyes on the tubercle bacillus outside the animal body has been tested several times on stained agar tubes to determine whether tubercle cultures would develop

TABLE 6.  
BACTERICIDAL ACTION OF TRYPAN BLUE ON TUBERCLE BACILLI, AS TESTED BY ANIMAL INOCULATION.

Dye Concentration	Quantity of Dye	Quantity of Bacillus Emulsion	Time of Exposure	Animal Used	Time after Injection before Tubercle Was Observed	Time after Injection before Death	Autopsy Findings
3 per cent. ....	5 c.c.	0.1 c.c.	5 minutes	Guinea-pig	15 days	78 days	Local and general tuberculosis
2 per cent. ....	5 c.c.	0.1 c.c.	5 minutes	Guinea-pig	15 days	85 days (then killed)	Local and general tuberculosis
1 per cent. ....	5 c.c.	0.1 c.c.	5 minutes	Guinea-pig	15 days	84 days	Local and general tuberculosis
0.1 per cent. ....	5 c.c.	0.1 c.c.	5 minutes	Guinea-pig	15 days	81 days	Severe local and general tuberculosis
Distilled water (control) ....	5 c.c.	0.1 c.c.	5 minutes	Guinea-pig	15 days	46 days (killed)	Marked local and general tuberculosis
3 per cent. ....	5 c.c.	0.1 c.c.	60 minutes	Guinea-pig	15 days	68 days	Severe local and general tuberculosis
2 per cent. ....	5 c.c.	0.1 c.c.	60 minutes	Guinea-pig	15 days	77 days	No autopsy
1 per cent. ....	5 c.c.	0.1 c.c.	60 minutes	Guinea-pig	15 days	83 days	Marked local and general tuberculosis
0.1 per cent. ....	5 c.c.	0.1 c.c.	60 minutes	Guinea-pig	15 days	77 days	Severe local and general tuberculosis
Distilled water (control) ....	5 c.c.	0.1 c.c.	60 minutes	Guinea-pig	38 days	53 days	Slight local, severe general tuberculosis
3 per cent. ....	5 c.c.	0.1 c.c.	20 hours	Guinea-pig	15 days	47 days (killed)	Severe local and general tuberculosis
2 per cent. ....	5 c.c.	0.1 c.c.	20 hours	Guinea-pig	15 days	74 days	Severe local and general tuberculosis
1 per cent. ....	5 c.c.	0.1 c.c.	20 hours	Guinea-pig	15 days	95 days (killed)	Severe local and general tuberculosis
0.1 per cent. ....	5 c.c.	0.1 c.c.	20 hours	Guinea-pig	15 days	78 days	Severe local and general tuberculosis
Distilled water (control) ....	5 c.c.	0.1 c.c.	20 hours	Guinea-pig	15 days	77 days	No autopsy
3 per cent. ....	5 c.c.	0.1 c.c.	44 hours	Guinea-pig	15 days	53 days	Severe local and general tuberculosis
2 per cent. ....	5 c.c.	0.1 c.c.	44 hours	Guinea-pig	25 days	78 days	Severe local and general tuberculosis
1 per cent. ....	5 c.c.	0.1 c.c.	44 hours	Guinea-pig	25 days	85 days (killed)	Severe local and general tuberculosis
0.1 per cent. ....	5 c.c.	0.1 c.c.	44 hours	Guinea-pig	25 days	92 days (killed)	Severe local and general tuberculosis
Distilled water (control) ....	5 c.c.	0.1 c.c.	44 hours	Guinea-pig	25 days	95 days (killed)	Severe tuberculous lymph adenitis. Liver and spleen not involved

in media colored with the dye in question. On account of the difficulty of securing uniform results by this method on so freakish an organism as the tubercle bacillus, inoculations have been made into guinea-pigs of material which had been subjected to the action of the dye for varying times. Trypan blue has been very thoroughly tested as to its bactericidal power over tubercle bacilli, and Table 6 gives in brief form the results of these tests. From this table, it may readily be seen that this dye has no appreciable power to kill these bacilli. In this experiment, as well as in those with the other dyes, the emulsion of tubercle bacilli was filtered and smears showed that there were no larger clumps, the bacilli being in the main single. This condition is of course most favorable for killing the organisms. One-tenth cubic centimeter of this filtered emulsion was added to five cubic centimeters of one per cent solution of

TABLE 7.  
BACTERICIDAL OR INHIBITORY ACTION OF DYES ON TUBERCLE BACILLI.  
A. *In vitro*.

Dyes Used	Amount of 1 Per Cent Dye Solution to 10 c.c. Agar Tube	Staining Result	Growth Result
Trypan blue.....	2 drops to agar tube	Good mass stain	Good growth
	4 drops to agar tube	No individual stain	
Trypan red.....	2 drops to agar tube	Good mass stain	Growth good
	4 drops to agar tube	No individual stain	
Rectified methylene blue	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Good individual stain	
Medicinally pure methylene blue.....	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Good individual stain	
Pharmacopeial methylene blue.....	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Good individual stain	
New methylene blue N.	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Good individual stain	
New methylene blue GG	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Good individual stain	
Brilliant cresyl blue.....	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Fair individual stain	
Gentian violet.....	2 drops to agar tube	No stain (1)	Good growth (1)
	4 drops to agar tube	Good mass and fair individual stain (2)	No growth (2)
Pyronin.....	2 drops to agar tube	Good mass stain (1)	Good growth (1)
	4 drops to agar tube	No individual stain (2)	Slight growth (2)
Neutral red.....	2 drops to agar tube	Only mass stain in both tubes	No growth in either tube
	4 drops to agar tube	Only mass stain in both tubes	
Janus green.....	2 drops to agar tube	Only mass stain in both tubes	No growth in either tube
	4 drops to agar tube	Only mass stain in both tubes	
Eosin A.....	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Fair individual stain	
Blue eosin.....	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Fair individual stain	
Erythrosin.....	2 drops to agar tube	Good mass stain	No growth
	4 drops to agar tube	Good individual stain	
Bismarck brown.....	2 drops to agar tube	Only mass stain in both tubes	No growth
	4 drops to agar tube	Only mass stain in both tubes	
Pyrrhol blue.....	2 drops to agar tube	Only mass stain in all tubes	Good growth in all tubes
Isamine blue.....	4 drops to agar tube		

TABLE 7—Continued.  
BACTERICIDAL OR INHIBITORY ACTION OF DYES ON TUBERCLE BACILLI.  
B. *In vivo*.

Dyes Used	Time of Exposure	Staining Results	Results in Animal
Trypan blue 0.1 per cent to 3 per cent water solution....	5 minutes to 44 hours	Good mass stain Faint individual stain	All animals developed tubercles in 15 to 25 days
Trypan red. 1 per cent water solution.....	24 hours	Good mass stain Faint individual stain	Developed tubercle in 19 days
Rectified methylene blue. 1 per cent water solution.....	24 hours	Both mass and individual stain good	No tubercle had developed after 55 days
Medicinally pure methylene blue. 1 per cent water solution.....	24 hours	Both mass and individual stain good	No tubercle had developed after 55 days
Pharmacopeial methylene blue 1 per cent water solution....	24 hours	Both mass and individual stain good	Tubercle observed in 25 days
New methylene blue N. 1 per cent water solution.....	24 hours	Mass and individual stain excellent	Tubercle observed in 25 days
New methylene blue GG. 1 per cent water solution.....	24 hours	Mass and individual stain good	Tubercle observed in 25 days
Brilliant cresyl blue. 1 per cent water solution.....	24 hours	Mass and individual stain fair	No tubercle after 55 days
Gentian violet. 1 per cent water solution.....	24 hours	Both mass and individual stain excellent	Tubercle observed in 35 days
Pyronin. 1 per cent water solution.....	24 hours	Only mass stain	Tubercle found in 19 days
Neutral red. 1 per cent water solution.....	24 hours	Only mass stain	Tubercle developed after 47 days
Janus green. 1 per cent water solution.....	24 hours	Only mass stain	Tubercle developed in 25 days
Eosin A.....	Not tested		
Eosin blue. 1 per cent water solution.....	24 hours	Good mass and individual stain	Tubercle developed in 25 days
Erythrosin. 1 per cent water solution.....	24 hours	Mass and individual stain excellent	Tubercle developed in 25 days
Bismarck brown. 1 per cent water solution.....	24 hours	Only mass stain	No tubercle after 55 days
Pyrrhol blue.....	Not tested		
Isamine blue.....			

dye, and at the end of the desired period, one drop of the mixed emulsion and dye was diluted with five cubic centimeters of sterile salt solution and injected subcutaneously into guinea-pigs. Table 7 summarizes both growth and animal experiments as to the power of the various dyes to kill the tubercle bacillus. In all the dyes except trypan blue, only one animal has been used, though the *in vitro* experiments have been repeated several times. In the one animal experiment, however, the time of exposure was 24 hours, and the other conditions were so severe that it seems reasonable to conclude that a given dye has little, if any, bactericidal power in case tuberculosis developed after the inoculation. In those cases in which no tubercle developed, however, no final conclusion will be drawn, but the experiment is being repeated on a larger number of animals. From this single experiment we can simply say that after 55 days' observation, no

tubercle had developed in the animals inoculated with tubercle bacilli exposed for 24 hours to one per cent solutions of Ehrlich's rectified methylene blue, medicinally pure methylene blue, brilliant cresyl blue, and bismarck brown, while with gentian violet and neutral red, the development of a tubercle was much delayed none being found until after 35 and 47 days respectively. All the others developed local tubercles within 15 to 25 days.

#### SUMMARY.

1. Among the dyes so far tested, trypan blue, trypan red, isamine blue, pyrrhol blue, Ehrlich's rectified methylene blue, medicinally pure methylene blue, methylene blue of the U.S. Pharmacopeia, new methylene blue N, new methylene blue GG, and to some extent neutral red and pyronin have been found to penetrate tubercles in guinea-pigs. Basic fuchsin, crystal violet, and the other new methylene blues are now being tested, and a report will follow later.

2. The dyes above mentioned are well borne for a long period, if the dose of the methylene blues, basic fuchsin, and crystal violet is not too large. Almost any dose of the first four dyes mentioned is well borne.

3. The individual bacillus itself is penetrated and well stained by all the methylene blues, by basic fuchsin, and crystal violet, by erythrosin and the eosins; not so well by trypan blue, trypan red, isamine blue, pyrrhol blue, pyronin, and neutral red.

4. Methylene blue, bismarck brown, and brilliant cresyl blue are the only dyes which have a possible bactericidal power over the organism, though many of the others seem to inhibit its growth in the test tube.

Such are the conclusions from the work actually done. Practically the only work published on the therapeutic value of dyes in tuberculosis is the work of Gräfin von Linden and her coworkers on the so-called "Finkler Heilverfahren" with methylene blue. The therapeutic use of methylene blue is by no means new, since Ehrlich states that it was recommended to him as an internal antiseptic by Leopold Landau in 1880, and later Ehrlich found it *nicht ganz erfolglos* in neuralgia, while still later the same worker found

it to have some specific influence on some cases of malaria. Its use in rheumatism, gonorrhea, and other infections is too well known to need mention. Gräfin von Linden claims that of the infected guinea-pigs treated with methylene blue chlorid or iodid, the local tubercles are healed in one-half to two-thirds of the animals, the temperature and weight and general condition are favorably influenced, and life is prolonged, in most cases the late death being due to some intercurrent infection. She also finds the dye penetrating the tubercle and staining the bacilli within the tubercle. Her clinical coworkers, Strauss and Meissen, also report some favorable results. While we have every reason to hope that methylene blue or some modification of that dye may have a favorable influence on tubercle bacillus infections, doubt is of necessity cast upon v. Linden's work, (1) on account of the small number of experimental animals treated with methylene blue, and (2) because Hugo Selter, originally one of her coworkers, states that the results are by no means conclusive.

My experiments with methylene blue have verified v. Linden's, so far as staining the tubercle bacilli *in vitro* and also penetrating the tubercle *in vivo*. I have, however, never found stained tubercle bacilli in the tubercle or in tuberculous pus stained with the dye.

I have for some weeks been treating tuberculous guinea-pigs with various preparations of methylene blue, but it is too early to make any report upon these, or even to judge as to whether any marked favorable influence is being exercised. I am also using therapeutically a number of other dyes and some modifications of trypan blue and allied dyes, which, it is hoped, will retain the favorable properties of trypan blue and at the same time have more marked bactericidal and curative properties. A report of these experiments will be published later.

It gives me great pleasure to acknowledge my indebtedness to Dr. H. Gideon Wells both for suggesting the work and for his constant and helpful interest in its progress.

## STUDIES ON THE GONOCOCCUS.\* I.

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The present study is based on the study of 15 strains of *Micrococcus gonorrhea* (Neisser) obtained from the urethral discharges of acute cases of gonorrhea in men. All primary cultures were made on ascites fluid agar or on rabbit blood agar.

### CULTURE MEDIA.

*Body fluid media.*—For the successful cultivation of the gonococcus slightly acid (+0.7 to 1.5) media, containing preferably human body fluids, such as ascitic or hydrocele fluid, blood or blood serum, are essential. I have been unable to substantiate the claims of Thallman,<sup>1</sup> Paldrock, and others as to the utility of simple media, urinary and otherwise. They are of no value whatever. Hydrocele or ascitic broth is of considerable value in the detection of contaminating organisms and in the preservation of cultures of gonococci for considerable periods of time. Contrary to the statements of some observers, the gonococci grow only on the surface of this medium. The sediment consists wholly of dead organisms and their debris. Blood agar, prepared both by admixture of blood before the agar solidifies and by smearing blood over the solid agar surface, is valuable, but less so than the ascitic or hydrocele agar. It is well to emphasize the importance of a slightly acid reaction in all media. It has been pointed out that human blood serum, hydrocele, and ascitic fluids may be alkaline to litmus and acid to phenolphthalein. In general, an acidity of +0.7 to 1.5 (10 c.c. medium requires 0.07 to 0.10  $n/10$  NaOH) will be found most satisfactory. It is only partly true that almost any alkaline medium is suitable for the growth of gonococci. Occasionally, abundant growths may be obtained on simple alkaline

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<sup>1</sup> *Centralbl. f. Bakt.*, 1900, I, 27, p. 828.



media, but such growths always show excessive autolysis and precarious stability, while more often there results no growth at all.

When blood is streaked on the agar surface the individual colonies present a characteristic and unmistakable appearance. In 24 hours they are less than pinhead size, gray-white, opaque, irregularly round, slightly convex, and with a  $\times 8$  hand lens they show a crumbly consistence and a finely stellate or radiate arrangement, the red blood having settled into the radiations like red spokes in a wheel. Very minute concentric striae or lamellae may be observed with a higher power. In examining such colonies it is well to hold the tubes up to a strong light and examine the colonies from the back. The blood should not be so thick in the medium as to prevent translucence. The separate colonies are easily picked off *in toto* with a fine platinum wire. Such colonies may attain  $\frac{1}{4}$ -inch size in 48 hours. Old colonies take on a glistening silvery sheen.

*Artificial serum medium.*—The effort to find some medium which, free from body fluid, would support life in gonococci led me to try innumerable combinations, with the result that one was hit upon which promised gratifying results. This consisted of a modified Ringer's solution, the formula for which follows:

Sodium chlorid.....	1.08
Potassium chlorid.....	0.045
Calcium chlorid.....	0.025
Sodium bicarbonate.....	0.020
Agar.....	0.250
Nutrient broth.....	20.00
Distilled water.....	100.00

The salts and agar are dissolved in the water by the aid of heat until the fluid shows a translucent ground glass appearance. To this is added the broth and the whole is filtered while hot through gauze or cotton into test-tubes or flasks. One sterilization in the autoclave is sufficient. The medium does not stand repeated Arnold sterilization as the agar is apt to be thrown out of suspension. When cool the medium is semisolid and has a translucent silvery appearance.

The limitations of this medium are that not all strains of gonococci will grow on it, nor is it easy to prepare, nor does it lend itself readily to centrifugation. The medium will, however, support all strains of gonococci, if there be placed on its surface a few loopfuls or a tiny drop of sterile human blood. The gonococci then thrive, while contaminating cocci and bacteria of all kinds except

the subtilis bacillus settle to the bottom of the tubes or flasks in trailing white, comet-like or stalactite-like festoons. The medium supports life in gonococci longer than any medium known to me, successful transplantations having been made after 100 days. It is also valuable for isolation of pure strains. It is by no means uncommon that individual gonococcus colonies on solid media contain contaminating organisms, so that pure strains are difficult to procure. Indeed, everyone who has worked long on the gonococcus knows this peculiarity and appreciates the difficulty of obtaining pure cultures. Not infrequently the procedure requires a struggle of weeks or months.

Beer-wort and media containing alcohol are of no value.

It is the observation of many bacteriologists that the gonococcus produces acid in dextrose media, but does not change saccharose, lactose, inulin, levulose, galactose, or mannite. Using ascitic broth to which the various carbohydrates were added in varying proportions and of which the reactions varied from slightly alkaline to +2.0, this predilection was confirmed. Titration of the medium after incubation at 37° C. for 14 days invariably showed increased acidity in the tubes containing glucose, but no changes with saccharose, inulin, or mannite. One strain out of 15 showed slight changes in tubes containing galactose.

The conclusions of Watabiki<sup>1</sup> were not confirmed, although horse serum which that author used as the diluent for his fluid media was not employed.

*Media containing organic substances.*—To ordinary nutrient agar and nutrient broth were added varying proportions of such animal substances as powdered mucin, fibrin, ovary, testicle, thyroid, pituitary, and lymph gland. Of media so prepared those containing ovarian and the testicular substance one to two per cent, slightly acid in reaction, proved excellent, while the others gave indifferent results. Vegetable substances, such as yeast nuclein, Irish moss, tragacanth, and acacia were used in a similar manner. Of these, acacia yielded good growths. The best combination proved to be a saturated solution of powdered acacia in nutrient agar, having a slightly acid reaction. The medium quickly

<sup>1</sup> *Jour. Med. Res.*, 1909, 20, p. 365.

becomes wrinkled, and is of no value aside from indicating a fondness of the gonococcus for the acacia enzyme. Certain animal substances containing enzymes were similarly employed, viz., pepsin, pancreatin, and ptyalin, pepsin and pancreatin yielding important and surprising results. The preparations of pancreatin used were the ordinary pancreatic substance (insoluble) and a purified pancreatin rendered soluble by acetone fractioning. Both of these preparations in one to two per cent strengths in slightly alkaline, neutral, and slightly acid nutrient agar influenced the growth of gonococci in a marked degree. In no medium was the growth so luxuriant and rapid as in the neutral pancreatic media, but the organisms at the same time appeared to undergo an extraordinarily swift autolysis. They appeared well-nigh structureless and stained very poorly with the strongest acid stains. Growth in the acid medium was somewhat slower, but still copious, and the organisms were better preserved. The growth is very short lived. Daily transplantations are necessary, and even then strains die suddenly. Growth is not invariable. Occasionally inoculations will not "take" at all, but the thick, opaque, yellowish-white, smeary, tenacious mass quickly covering the entire surface of the medium is quite characteristic.

Soluble pepsin in the same proportions and reactions also yielded abundant growths, but with a slightly acid reaction, quite rapid and showing much better viability and staining reaction, and less autolysis. Like pancreatin, it yielded irregular results, but one is inclined to the opinion that all irregularities in "takes" might have been due to variations in technic rather than to special properties of media or strains, since the same irregularities are to be observed with other media routinely employed. Not enough work has been done with these enzymes to warrant any conclusion but the results indicate a predilection of the gonococcus for certain enzymes and an antipathy for others. Sterile preparations of pepsin and pancreatin are difficult to obtain. Dry heat just short of  $100^{\circ}\text{C}$ . changes the color of the products although it is said not to destroy the activity of the ferments. Boiling destroys the enzyme. Good results were obtained by heating solutions of pepsin and pancreatin to  $60^{\circ}\text{C}$ . for one hour on three successive

days. These substances are distinctly acid in reaction and require the addition of alkali, preferably NaOH solution, before being added to culture media. The addition of alkali also renders the substances more readily soluble. Further mention of the enzyme relationships will be made later in this paper and, it is hoped, more fully in a subsequent communication.

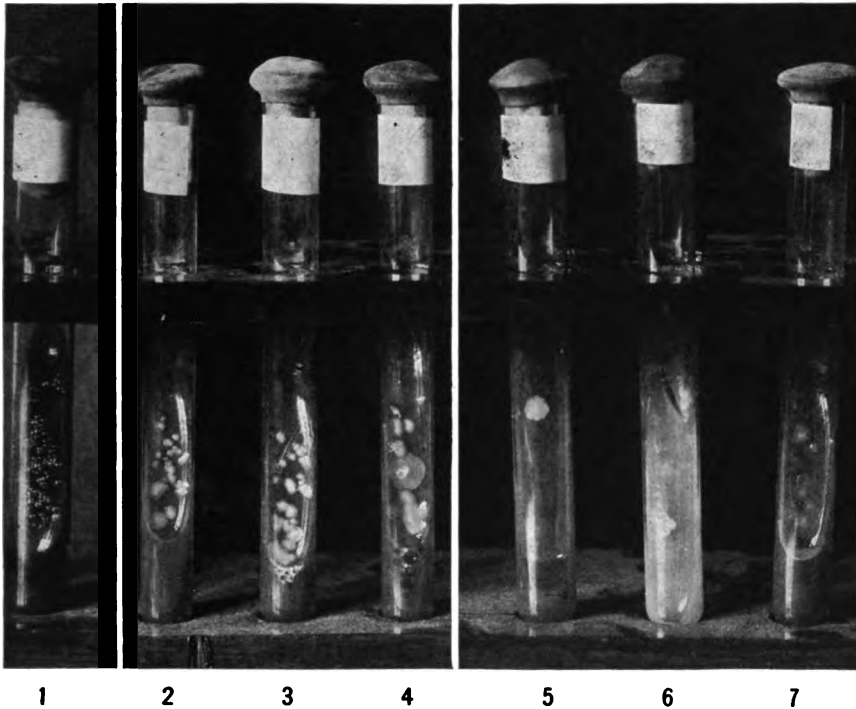


FIG. 1.

- 1.—Gonococcus colonies on rabbit blood agar from heart's blood of guinea-pig, five hours after intraperitoneal inoculation.
- 2.—Meningococcus colonies on pancreatic agar.
- 3.—Gonococcus colonies on pepsin agar (+r.o.).
- 4.—Gonococcus colonies on pancreatic agar. (5 c.c. nutrient agar—0.020 solution pancreatic. Reaction +r.o.)
- 5.—Meningococcus colony on alkaline pancreatic agar.
- 6.—Gonococcus colony on alkaline pancreatic agar.
- 7.—Gonococcus on nutrient pancreatic agar.

Stab cultures and anaerobic culture media in general are not favorable to growth of gonococci, although unquestionably some

growth does occur, provided the media contain the ascitic fluid or other favorable substance. This we should expect to be the case from our knowledge of the pathology of gonorrheal infections. The organisms survive for considerable periods of time without air. Voluminous cultures upon ascitic agar placed in a vacuum chamber over calcium chlorid and kept at 20° C. for two weeks survived and subcultures were successful.

#### MORPHOLOGY.

The morphology of the gonococcus is so variable as to have given rise to considerable discussion. My experience has been that by using many strains and by varying the media on which they are grown, one can observe all the types of the entire coccus family. By undoubted processes of reversion and variation it is possible, and common, to observe staphylococcus-like clumping of single forms, short chain formation as with the streptococci, lanceolate forms indistinguishable from pneumococci in shape, diplococcal forms resembling the catarrhalis groups, all in unquestionably pure cultures of gonococci. All return promptly to strain type on appropriate media. For instance, the strains grown in fluid media show the most lanceolate and chain forms; those grown on an over dry medium contain the larger number of clumps, while the cocci bred on moist ascitic agar of definite salt content and uniform reaction yield the fewest variants. From the examination of many hundreds of cultures and thousands of preparations, my opinion concurs with the view that the gonococcus is classed properly as a micrococcus. A majority of the forms are single, irregularly round and discrete. Diplococcal forms, biscuit or coffee-bean in shape, follow numerically and appear to denote active proliferation. Still a larger number of cocci in all cultures show variation and irregularity. Variations in size are common in different media. By varying the salt content of ascitic agar, for instance, one is able to breed cocci of all sizes from the small staphylococcus size to those having a diameter equal to half that of a red blood-cell. The largest cocci were produced on salt-free ascitic agar, the smallest and most stable organisms on human blood agar; among

the larger cocci were many ring forms with the staining portion at the periphery and the centers shadowy.

The gonococcus is difficult to stain well. After having used a multitude of ordinary and special stains, it was found that the sharpest contours and the most brilliant staining are obtained with carbol fuchsin, one part of the ordinary tubercle carbol fuchsin and three of distilled water. Strangely enough, the methylene blue stains are very unsatisfactory for work with gonococcus cultures. The organisms do not take the stain deeply and the contours are not clear cut or sharply defined. The variations in character of different strains will embrace all variants, even for complement-fixation work.<sup>1</sup> My own observation would designate six selected strains as sufficient to cover all characters. The usual variations in appearance, consistence, colony forms, etc., are too well known to require further comment. The best descriptions and illustrations of cultures and organisms are by Martin.<sup>2</sup>

#### AUTOLYSIS.

It has been a matter of common observation for some time that gonococci suspended in water and in salt solution disintegrate with more or less rapidity, so that eventually no organisms whatever can be distinguished. To this peculiarity has been given the name autolysis, which is particularly apt since the property of solution or disintegration seems to be one inherent with the gonococcus. The autolysis is quite comparable to that which Rosenow and others have demonstrated in the pneumococcus. That the substance which causes the solution of the bodies of the cocci is itself a product of their life and activity is shown by the fact that autolysis occurs when all other possible causes have been removed. Gonococci which have been washed three times in distilled water, once in 0.125 NaOH solution, once in 1:5000 HCl solution, will, when again suspended in salt solution or distilled water, disintegrate with the same rapidity as unwashed cocci freshly removed from the culture medium. No culture or colony of gonococci is so young but that autolysis has already begun. Observation has

<sup>1</sup> Torrey, *Jour. Med. Res.*, 1907, 16, p. 329, and 1908, 19, p. 471; Schwartz and McNeil, *Am. Jour. Med. Sci.*, 1911, 141, p. 693.

<sup>2</sup> *Jour. Path. and Bact.*, 1911, 15, p. 76.

shown that the life history of the individual gonococcus is, in its own sphere, comparable to the ephemerae. Within an amazingly brief period the sequence of coccus, diplococcus, and disintegration occurs. As each organism dies it contributes a heritage which is cumulative and hastens the death of its fellows until within the space of a few days a culture of untold millions of the cocci has perished, destroyed by the combined activities of its individuals. In transplanting cultures over 48 hours old larger quantities must be transferred in order that the few live cocci therein may be included.

*Agents which check autolysis.*—McClintock and Clark<sup>1</sup> have shown that a temperature of 70° C. for one hour effectually checks disintegration of suspensions of gonococci in salt solution. Temperatures less than 70° C. with or without the addition of tricresol 0.2 to 0.4 per cent do not avail.

Hydrochloric acid solution in distilled water in strength as low as 1:5000–1:10000 is the best preservative of gonococci. Suspensions of gonococci in the medium remain unchanged for months, and serve as the most stable vaccine that has so far been prepared. Tricresol may be added if desired, preferably a strength of 0.2 per cent. The gonococci are quickly killed in the solution. Organisms preserved for months in this manner resume autolysis upon washing and suspending in salt solution. Hydrochloric acid solution produces a strong agglutination of the gonococci, a fact which necessitates breaking up of the clump by shaking with glass beads, in the preparation of vaccine.

Lactic, acetic, nitric, benzoic, and oxalic acids in weak solutions in distilled water are also preservatives of the bodies of gonococci. It appears that almost any weak acid tends to overcome autolysis.

Glycerin solution five per cent in distilled water is a good preservative for short periods of time. Pepsin, 0.5 neutral, or slightly acid solution in distilled water is almost as good a preservative as dilute hydrochloric acid, but a slight yellowish-brown discoloration occurs on standing for a considerable time. Heavier pepsin percentages cause digestion.

Alcohol solutions as high as 10 per cent, weak solution of formalin, chloroform water, all sufficiently strong to prevent bacterial growth, appear to have little or no influence upon autolysis, providing the reaction of the solution is neutral or slightly alkaline. In conducting experiments on digestion one not infrequently adds some substance like chloroform, which prevents bacterial growth, but in no way hinders the activity of the digestive enzyme, and it is noteworthy that the same substances do not prevent the autolysis of gonococci.

*Agents which favor autolysis.*—Salt solution 0.8 to 0.9 per cent and distilled water are very favorable agents. If to either there be added  $n/10$  NaOH solution or other alkali sufficient to produce slight alkalinity, it is observed that autolysis is materially hastened. Normal blood alkalinity (0.125 approximately) is a very favorable degree. It was also observed that addition of small quantities of those proteolytic enzymes

<sup>1</sup> *Jour. Infect. Dis.*, 1909, 6, p. 217.

which operate in an alkaline medium hastens lysis of the gonococci. In particular, it was found that the most rapid lysis of all occurred when the organisms were suspended in a slightly alkaline solution of pancreatin and preserved at 37° C. Beautiful illustrations of the disintegration may be observed in hanging-drop preparations where the process in a single field may be watched, and in stained preparations made from hour to hour.

*Digestive powers of autolysates and dried gonococci.*—Autolysates of gonococci were prepared by suspending 24-hour cultures in 0.9 per cent salt solution, allowing the suspension to stand at room temperature for three days, shaking with quartz sand for three hours, after which the debris was removed by passing the fluid through a Berkefeld filter. The filtrate had marked toxic effects on rabbits and guinea-pigs, three cubic centimeters being sufficient to kill a rabbit of 1,500 gms. in half an hour.

Gonococci were dried by suspending larger cultures in as small a quantity of distilled water as practicable and evaporating the fluid in a vacuum chamber. The dried substance is light brown in color. Cultures from 12 whiskey-flask surfaces yield a little over one gram of dried substance. These are also highly toxic.

With these substances the following digestive test was made:

- Controls:* 1. Pancreatin solution 0.020; salt solution 4 c.c.  
2. Gonococcus autolysate 4 c.c.  
3. Dried gonococci 0.020; salt solution 4 c.c.  
4. Fibrin 0.020; salt solution 4 c.c.

*Determinants:* 1. Pancreatin 0.020; salt solution 4 c.c.; fibrin 0.020, alkaline to litmus.

2. Gonococcus autolysate 4 c.c.; fibrin 0.020, alkaline to litmus.  
3. Gonococcus autolysate 4 c.c.; fibrin 0.020, acid to litmus.  
4. Dried gonococci 0.020; salt solution 4 c.c.; fibrin 0.020, acid to litmus.  
5. Dried gonococci 0.020; salt solution 4 c.c.; fibrin 0.020, alkaline to litmus.

To each tube was added a few drops of chloroform and all were placed in the incubator at 37° C. for eight hours.

*Result:* Controls 1, 2, 3, and 4—Negative Biuret.

- Determinants 1 = +Biuret  
2 = ++Biuret  
3 = +Biuret  
4 = ++Biuret  
5 = +Biuret

It seems reasonable to assume that the gonococcus produces a proteolytic enzyme, similar to trypsin, which has a temperature range of viability between 0° C. and 70° C. It will be shown later that the physiological effects of the enzyme on laboratory animals are very strikingly similar if not identical with those of trypsin. It appears that the enzyme is capable of slight activity in an acid medium although a slight HCl acidity is capable of preventing its formation. It is a question whether this substance or enzyme can be classed with the toxins in the sense that we refer to the



soluble toxin of the diphtheria bacillus and the endotoxin of the typhoid bacillus. The so-called toxins of the gonococcus, the meningococcus, and the pneumococcus, and perhaps also the streptococcus appear to form a group by themselves. Certainly those of the gonococcus and the pneumococcus present strikingly similar features.

#### AGGLUTINATION.

Tests of agglutination of gonococci with antisera have been disappointing in my hands. The claims of many to have produced sera which will agglutinate in dilutions of 1:50000-1:200000 I have been unable to substantiate. The most powerful serum in my series was that of a rabbit which had received three inoculations only, as follows:

February 5, 1912: Intravenously, the autolysate of one ascitic agar tube of gonococci kept for 10 days at 37° C. in two cubic centimeters of rabbit serum.

February 20, 1912: Intraperitoneally, one large loop of 24-hour ascitic agar culture of gonococci in two cubic centimeters of sterile salt solution.

February 27, 1912: Intraperitoneally, one ascitic agar tube culture suspended in two cubic centimeters sterile salt solution.

The animal was bled for serum five hours after the last inoculation.

April 20, 1912: Agglutination test.

Standard suspension of gonococci in distilled water.

Serum dilutions: 1:1 = + at once

1:1000 = + at once

1:50000 = + in one hour.

By way of comparison, a control agglutination test was made with an antigonococcus serum (017326 K. B 1095) on the same day. The results were as follows:

Dilution 1:10 = negative in two hours; partial in 18 hours.

Dilution 1:40 = negative in two hours; slight in 18 hours.

Antimeningococcus serum (018781) gave a positive agglutination of gonococci in the dilution of 1:10 in two hours.

Antidiphtheric serum, normal horse serum, and certain guinea-pig sera give positive results in eight hours' time, but almost any serum will do the same, whereas those sera produced by long-continued immunization of rabbits with large quantities of gonococci do not show that degree of agglutinative power one would expect. For instance, one rabbit received in all 16 inoculations, the first 12 intravenously at intervals of five days, the last four intraperitoneally at 10-day intervals. This serum was positive in a dilution of 1:10 in three hours and negative in a dilution of 1:40.

The substance which invariably produces the most marked agglutination of gonococci is HCl in dilution of 1:2500 and as high as 1:10000 in distilled water. Salt solution with HCl in the latter dilution is not favorable and, indeed, permits some autolysis.

*Gonococcidal substances.*—All ordinary germicidal substances are fatal to the gonococcus. Indeed, distilled water is destructive and salt solution even more markedly so. On the most favorable culture medium the life of the organism is very short at best. It was impossible to determine whether the silver salts, those of mercury, the acids, the alkalies, formol, the essential oils, the halogens, all in the strength usually employed clinically, were fatal, one sooner than the other, since all were quickly fatal. The experiments of Wildbolz,<sup>1</sup> Paldrock, and others upon the viability of the gonococcus in germicidal solutions are valuable and of great interest, but the brief periods of time, even to the fractions of minutes mentioned by some, reveal what a painstaking procedure is demanded.

#### ANIMAL EXPERIMENTS.

All efforts to produce a specific inflammation of the eyes or genitalia of animals were futile, as were the efforts to produce abscess, by inoculating animals with fresh cultures of gonococci.

The mechanism of defense of the mucous surfaces of all animals is much the same. The eye of the rabbit furnishes a good example. Five minutes after the conjunctival sac has been smeared with freshly isolated pure 24-hour cultures of gonococci there appears at the inner canthus a globule of grayish exudate which is seen in stained preparations to consist of mucus and immense numbers of epithelial cells, clinging to which are larger clumps of gonococci which stain poorly and appear to be undergoing rapid lysis. There are but few polymorphonuclear leukocytes. After 15 minutes the picture is much the same except that the cocci are much diminished in number. In 30 minutes the exudate has become more watery and consists of epithelial cells and leukocytes, but evidence of phagocytosis is wanting. In an hour, all cocci have disappeared and the eye appears normal. At no time does the eye show inflammatory reaction. All efforts to hold the culture in place in the conjunctival sac by mechanical means failed. The submucous injections of cultures were also negative.

The few positive results obtained in animals will be briefly outlined.

*Monkeys (M. rhesus) small; 7 pounds weight.*—No. 1. Hypodermic injection of morphin sulphate 2 grains; intradural injection of one-half tube of ascitic agar 24-hour culture suspended in 2 c.c. normal monkey serum. Elevation of temperature (104.5° F.), coma, convulsions, and death in nine hours. Autopsy showed local fibrin-like exudate at site of inoculation and reddening of the meninges. Cultures negative. Exudate showed a few poorly stained shadowy cocci, leukocytes, and debris; no organisms.

<sup>1</sup> *Arch. f. Dermat. u. Syph.*, 1903, 64, p. 471.

In the left conjunctival sac of the same monkey there was placed one-half tube of ascitic agar tube culture 24 hours old on the end of a spud synchronously with the intradural injection. Postmortem appearances: right eye normal; left eye protruded, lids everted and swollen, conjunctiva chemotic. Dirty gray exudate in moderate amount containing leukocytes, epithelial cells, and debris. Smears and cultures negative for gonococci. Sections of lower lid and conjunctiva showed inflammation and edema but no organisms.

No. 2. Morphin sulphate 2 grains. Intravenous injection of one-half tube of ascitic agar tube culture of gonococci, 24 hours old, in two cubic centimeters salt solution. Elevation of temperature (106° F.), coma, death in 12 hours. Endocardium over chambers and valves appeared lusterless and "smoked." Smears from liver and spleen showed poorly stained, irregular, shadowy cocci. Phagocytosis absent. Cultures from heart's blood on ascitic and blood agar positive.

No. 3. (Control)—Morphin alone. Recovered.

No. 4. (Control)—Intravenously one-half ascitic agar tube culture in two cubic centimeters salt solution. Temperature maximum 104° F. Recovered.

No. 5. (Control)—Intraperitoneally, one-half ascitic agar tube culture in two cubic centimeters salt solution. Recovered.

*Rabbits*.—It was demonstrated that phagocytosis plays small part in the immunity of these animals, as well as in other laboratory animals. This statement is based on a large number of examinations of blood and peritoneal exudates in inoculated animals, and in many phagocytic experiments *in vitro* with the blood of animals.

Rabbits react to both intravenous and intraperitoneal injections of gonococci, with prompt rise of temperature, which quickly returns to normal.

*Guinea-pigs*.—Guinea-pigs of all laboratory animals manifest the nearest approach to a true septicemia following inoculations with cultures of gonococci. The response is prompt and definite and in every instance where a fatal dose was given, gonococci were recovered in cultures from the heart's blood and the organisms were demonstrable in smears from the liver, spleen, and lungs. Phagocytosis is not a conspicuous feature, although it appears to be somewhat more common in this species than in others. The initial fatal dose of most strains is the 24-hour growth of one ascitic or blood agar tube. This was suspended in two cubic centimeters of sterile salt solution and injected into the peritoneal cavity. The animal is at once ill, and the temperature begins to fall and continues to fall steadily until the animal dies in from five to 12 hours. Animals surviving longer than 12 hours usually recover. The peritoneal exudate after one hour is serous and tinged with blood. It contains numerous red cells, innumerable well staining gonococci, and only few leukocytes of any kind. The few polymorphonuclear cells observed seldom contain cocci. After three or four hours, and after death, the fluid exudate, about 3 to 5 c.c. in amount, is grayish and contains flakes of lymph. The peritoneum is flecked with lymph, particularly about the liver and over the intestine. At this time the exudate contains greater abundance of leukocytes, but still the polymorphonuclear cells are few and phagocytosis is not conspicuous. Those cocci which live within the cells are ragged, misshapen, and do not take the stain well. Gonococci are still numerous but are rapidly diminishing in numbers and losing their outlines. The liver and spleen are usually dark red, enlarged, and congested. Cocci are always demonstrable in smears from the pulp of these organs, being for the most part free while a few poorly stained organisms are seen within the leukocytes. Cultures from the heart's blood are almost invariably positive, and from this fact it was

learned that the quickest and surest method of purifying contaminated strains of gonococci is by injecting the culture in large quantities into guinea-pigs intraperitoneally, and inoculating the heart's blood on blood agar five hours later, after chloroform death.

The strain of gonococci resulting from such cultures was used over and over again, passing it from tube to pig and pig to tube regularly every 24 hours for 30 days. The strain then appeared to have lost some of its autolytic tendency; it took the ordinary stain better, became more hardy and adapted itself readily to ordinary nutrient agar (reaction  $+0.7$ ), on which medium it survived eight days at  $37^{\circ}$  C. and was carried on through 22 subcultures. The impression was gained that the gonococcus is capable of modification by environment into an organism of fair stability, in spite of the observation that continued operation of the same strain through guinea-pigs did not appear to lessen the quantity required for a lethal dose.

## FIXED HOG CHOLERA VIRUS.\*†

JOHN REICHEL.

(From the Mulford Laboratories, Glenolden, Pennsylvania.)

Hog cholera is at present correctly classed as one of the invisible-virus diseases. Little has been added to the work of Dorset, Bolton and McByrde<sup>1</sup> in which they proved the specificity of the virus. Aside from knowing that it is invisible, filterable, demonstrable only by injecting suspected material into susceptible hogs, easily destroyed by heat, and unusually resistant to some of the well-known disinfectants we have no clue to its real nature. The work of Dinwiddie<sup>2</sup> presented at the 49th annual meeting of the American Veterinary Medical Association encourages the hope that the tissue-element of the red blood corpuscles of the hog may perhaps be demonstrated as the host of the specific cause.

Judging from the natural outbreaks of the disease, the variation in the virulence of virus is a very important characteristic, upon which preventive measures including the success in the immunizing and curative treatment with hog cholera serum largely depends. In preparing virus, or virulent blood, for 1,181 hyperimmune hogs, 1,964 supposedly susceptible hogs were injected subcutaneously with from two to five cubic centimeters of virus, and only 144, or 7.3 per cent, resisted infection. The 1,720, or 93.7 per cent, all developed the disease in less than 40 days.

The virus hogs were kept in regulation virus-pens—8 by 10 feet, elevated 18 inches from the ground on four legs, with waterproof metal-lined floor, slant roof, both sides solid, small narrow drop-door for ventilation in back, with door and two windows in front. Each pen is equipped with a wooden feeding trough and card holder to accommodate a card for each hog. The pens are placed in two rows of five each, from four to five feet apart. The two rows are

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† Read at the meeting of the United States Livestock Sanitary Association, December 4, 1912.

<sup>1</sup> Bureau of Animal Industry, U.S. Dept of Agriculture, *Bull. No. 72*.

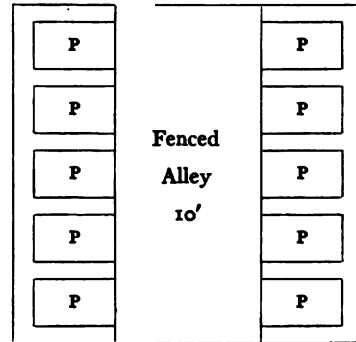
<sup>2</sup> *Report of 49th Annual Meeting of the Amer. Veter. Med. Assoc.*

separated by a fenced alley approximately 10 feet wide. The group of pens in turn is surrounded by a fence which prevents all communication from pen to pen except by the door-way of each pen.

Although 19 strains are listed in Table 1, not all of the strains were continually passed through susceptible hogs. In fact no more than six were passed through hogs in the virus-pens at any one time.

The table records 13 separate but not consecutive months. In fact a number of the strains listed were acquired several years ago, and when not passed through hogs during any one month, the "seed" of the strain was kept in sealed bulbs at a low temperature, and the month was not counted in the tabulation. A strain, passed through hogs during a number of separate

OUTLINE OF VIRUS-PEN  
INCLOSURE



but not consecutive months, was injected into not less than one lot of hogs each month and during that month the strain may have been passed through two or more hogs. Although the hogs were injected for the chief object of producing virus or virulent blood for the injection of hyperimmune hogs every effort was made to increase the virulence of the strains and to keep them pure, so that the stronger would not supplant the weaker. All the hogs for the production of virus were purchased by one who was thoroughly familiar with our needs. That they should be corn-fed and from hog-cholera-free sections of the country were chief requisites; that susceptible hogs were supplied is proved by the fact that 93.7 per cent succumb. If, as happened several times, there was reason to believe that the hogs were infected upon arrival, no "seed" was saved to carry the strain from the suspected lot.

Upon the arrival of a lot of hogs for the virus-pens, each hog was injected with from two to five cubic centimeters of the seed-virus, and placed in the virus-pen assigned to the strain. The pens were not cleaned during the time each lot of hogs was kept in them. Grain was fed twice daily and water placed in the feed troughs

TABLE 1.  
PASSAGE OF HOG-CHOLERA VIRUS THROUGH SUSCEPTIBLE HOGS.

Virus	Source	1st Month	2d Month	3d Month	4th Month	5th Month	6th Month	7th Month	8th Month	9th Month	10th Month	11th Month	12th Month	13th Month
V 1	North Dakota.....	46*	29	54	49	37	11	14	9	6	....	....	....	....
		9.5†	10.9	7.7	7.5	11.5	17.8	7.6	9.7	11.3	..	..	..	..
V 2	Minnesota.....	21	5	49	36	30	12	10	17	16	..	..	..	..
		7.2	8.8	9.3	5.4	7.1	7.7	7.1	10.6	6.7	..	..	..	..
V 3	Pennsylvania.....	43	6	3	16	29	10	4	8	3	..	..	..	..
		19.4	4	10.3	7.2	6.5	6.4	7.2	9.3	10.3	..	..	..	..
V 4	Pennsylvania.....	6	6	7	9	8	8	5	3	6	..	..	..	..
		14.4	8.6	6.4	5.9	5.5	9.2	8	6.6	5.6	..	..	..	..
V 5	North Dakota.....	8	83	6	1	12	5	5	6	..	..	..	..	..
		12.1	12.2	19.5	15	9.9	6.6	11.2	7.5	..	..	..	..	..
V 6	Ohio.....	6	2	18	7	2	5	5	..	..	..	..	..	..
		17.6	8.5	9.2	10.2	6.5	6.6	5.2	..	..	..	..	..	..
V 7	Pennsylvania.....	4	..	..	..	..	..	..	..	..	..	..	..	..
		7.7	..	..	..	..	..	..	..	..	..	..	..	..
V 8	Minnesota.....	1	9	..	..	..	..	..	..	..	..	..	..	..
		8	7.8	..	..	..	..	..	..	..	..	..	..	..
V 9	Minnesota.....	51	15	..	..	..	..	..	..	..	..	..	..	..
		8	6.3	..	..	..	..	..	..	..	..	..	..	..
V 10	Minnesota.....	7	..	..	..	..	..	..	..	..	..	..	..	..
		6.8	..	..	..	..	..	..	..	..	..	..	..	..
V 11	Delaware.....	29	28	18	31	6	..	..	..	..	..	..	..	..
		40.1	27.1	10.3	11.2	9.4	..	..	..	..	..	..	..	..
V 12	Ohio.....	5	4	..	..	..	..	..	..	..	..	..	..	..
		14.2	15	..	..	..	..	..	..	..	..	..	..	..
V 13		25	64	11	16	25	4	59	1	18	179	121	120	37
		30.1	21.2	20.5	30	36.2	12	13.4	5	19.2	11.4	20.4	14.1	21.3
V 14	Pennsylvania.....	3	..	..	..	..	..	..	..	..	..	..	..	..
		22.3	..	..	..	..	..	..	..	..	..	..	..	..
V 15	Kansas.....	11	58	11	..	..	..	..	..	..	..	..	..	..
		8.5	9.2	7.8	..	..	..	..	..	..	..	..	..	..
V 16	New Jersey.....	15	..	..	..	..	..	..	..	..	..	..	..	..
		14.2	..	..	..	..	..	..	..	..	..	..	..	..
V 17	New Jersey.....	20	..	..	..	..	..	..	..	..	..	..	..	..
		10.2	..	..	..	..	..	..	..	..	..	..	..	..
V 18	Missouri.....	2	..	..	..	..	..	..	..	..	..	..	..	..
		6.5	..	..	..	..	..	..	..	..	..	..	..	..
V 19	Missouri.....	6	..	..	..	..	..	..	..	..	..	..	..	..
		9.6	..	..	..	..	..	..	..	..	..	..	..	..

\* Number of susceptible hogs injected.

† Average number of days in which hogs developed hog cholera before being killed for virus or before dying.

without entering the pens. Temperatures, however, were taken and recorded daily. This was usually intrusted to one man who went from one pen to the next. Therein lies the possibility that infection was carried from one pen to the other, but a careful study of the table shows that any increase in virulence was gradual. Moreover, the care exercised in selecting the strain for "seed" reduced to a minimum the possibility that a weak strain was supplanted by a stronger.

According to the table the 19 strains had their origin in natural outbreaks in eight states. The top figures in each square opposite the number of each strain includes the number of hogs injected during each month; the lower figure gives the average number of days from the time the hog was injected until the disease had sufficiently developed for us to kill the hog for virus or virulent blood. Sudden deaths, before the hog was bled, are included as hogs killed.

With the exception of virus No. 1 the strains show a gradual increase in virulence, and we believe that we have had some of the strains under observation sufficiently long for us to regard them as having attained the maximum virulence possible. In other words, that these strains have approached or are rapidly approaching what Pasteur, working with rabies virus, called a "fixed virus." These strains are capable of producing the first symptoms—loss of appetite, occasionally reddening of the skin, and rise of temperature on the fifth or sixth day, with death on the seventh or eighth day as a direct result of subcutaneous injection in two to five cubic centimeter doses. The hogs killed in less than five days, included in the table, were probably of the small class infected before arrival.

It is interesting to note at this time that fixed rabies virus will bring about symptoms of the disease in rabbits following subdural injection on the sixth or seventh day, and death on the eighth or ninth day. The period of incubation of fixed strains of the so-called invisible viruses may be quite similar in length.

The virus or virulent blood collected from 1,720 hogs was entirely used up in hyperimmunizing 1,181 hogs of which only 6.7 per cent received one or more intraperitoneal injections and 2.7 per cent one or more subcutaneous injections. All the injections were



made intravenously except in the percentage referred to, in which cases intraperitoneal or subcutaneous injections were alone possible.

Virus propagated in the manner outlined above and injected intravenously will yield a uniformly potent serum, as has been proved by the accepted standard test which is as follows: Six susceptible pigs each weighing not less than 50 pounds are injected with doses of two cubic centimeters of virus, fixed virus being invariably used; two are then injected with 15 cubic centimeters and two with 20 cubic centimeter doses of the serum under test, and two are left untreated with serum, to serve as controls or checks on the virulence of the virus used and the susceptibility of the pigs. The virus used should kill the controls in at least 21 days while the serum should save the pigs treated.

## ON THE ORIGIN AND ACTION OF HEMOLYTIC COMPLEMENT.\*

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The rôle of complement in bacteriolytic processes is familiar. Recent investigations emphasize the importance of the complement of the blood in the parenteral digestion of proteins and in infections. Thus Friedberger and his associates<sup>1</sup> produced anaphylatoxic substances from bacterial and other proteins by means of the action of complement. Variations in the complement content of the blood during disease have been noted by Moro,<sup>2</sup> who concluded that a capacity for the ready formation of complement was of prognostic value. I have found<sup>3</sup> a decrease in hemolytic complement before the crisis and an increase at the time of crisis in pneumonia. Lüdke and others<sup>4</sup> found a decrease in complement in chronic suppurative processes. Eliasberg<sup>5</sup> found the blood of leprosy patients poor in complement. In view of the possibility of raising the complement content of the blood for therapeutic purposes, it is rather surprising that there is so little unity of opinion either as to the origin of complement or as to the nature of the chemical processes with which it is concerned.

### THE ORIGIN OF COMPLEMENT.

Since Buchner and Metchnikoff advanced the idea that complement was derived from the leukocytes, that idea has been the prevalent one, although there has been a great deal of experimental evidence both for and against it. Other locations which have been considered as a source of complement are the pancreas and liver.

1. *The leukocytes*.—The following points are advanced as evidence for and against the leukocytes as a source of complement:

a) Complement does not exist free in the plasma as it is removed from the body (Gengou<sup>6</sup>). The most recent confirmation of this evidence is by Gurd.<sup>7</sup> Contrary

\* Received for publication December 7, 1912.

<sup>1</sup> *Ztschr. f. Immunitt.*, 1911, 2, p. 471.

<sup>2</sup> *Über das Verhalten hemolytische Serumstoffe beim gesunden und kranken Kind*, Wiesbaden, 1908.

<sup>3</sup> *Jour. Infect. Dis.*, 1912, 10, p. 383.

<sup>4</sup> Quoted by Sachs, *Handbuch der Technik und Methode der Imm. Forsch.*, Gustav Fischer, Jena, 1909.

<sup>5</sup> *Deutsch. med. Wchnschr.*, 1901, 37, p. 302.

<sup>6</sup> *Ann. de l'Inst. Past.*, 1901, 15, p. 232.

<sup>7</sup> *Jour. Infect. Dis.*, 1912, 11, p. 225.

evidence is brought forward by a number of investigators. The work of Addis<sup>1</sup> seems especially convincing.

b) Serum left in contact with leukocytes (blood-clot) increases in complement content (Walker,<sup>2</sup> Smith,<sup>3</sup> Longcope,<sup>4</sup> and Gurd<sup>5</sup>). Of those whose work tends to disprove this evidence Addis<sup>1</sup> may be again cited.

c) Extract of organs containing many leukocytes acts as complement (Tarashevitch<sup>7</sup>). This is denied by Korshun and Morgenroth,<sup>8</sup> who were able to obtain a hemolysin from organ extracts, but this hemolysin did not act as complement as it was inhibited in its action by amboceptor.

2. *The pancreas*.—Sweet<sup>9</sup> concluded that after extirpation of the pancreas in dogs, the complement content fell. This conclusion, however, was based on observations made on the serum of one animal. This serum was removed after death and the complement content was not directly estimated, but the total lytic power was found to be decreased. On account of the fact that no diminution in amboceptor could be ascertained, the decrease in total lytic power was thought due to a decrease in complement.

3. *The liver*.—Müller<sup>10</sup> obtained an increase in both amboceptor and complement in the blood by means of injections of iodipin and attributes the action of this drug to an influence on the liver. His reason for regarding the liver as the source of complement is that serum perfused through the liver became stronger in complement. Recently Gay and Rusk<sup>11</sup> have been unable to obtain any increase of antibody production with iodipin.

In view of these conflicting ideas, it was thought desirable to make an investigation of the following points: (1) The presence of complement in plasma; (2) the influence of the blood-clot and its components on the complement content of serum left in contact with it; (3) a comparison of the complement in the fluid from leukocytic exudates with that of the blood serum.

In the complement estimations the following plan was used. An antisheep rabbit serum inactivated for one-half hour at 56° C. was used as an amboceptor. This was first titrated to determine the minimum dose required to cause lysis of a 5 per cent suspension of washed sheep erythrocytes with one-tenth cubic centimeter of 1:10 dilution of normal guinea-pig serum. The total volume of the mixture was always one cubic centimeter. This minimum dose was considered as one unit of amboceptor. A comparison was then made of the amounts of test and control serums required to give the same amount of hemolysis using 10 units of amboceptor.

<sup>1</sup> *Jour. Infect. Dis.*, 1912, 10, p. 200.

<sup>2</sup> *Jour. Hyg.*, 1903, 3, p. 52.

<sup>3</sup> *Proc. Royal Soc.*, 1906, 79, p. 378.

<sup>4</sup> *Med. Bull. Univ. of Penna.*, 1902.

<sup>5</sup> *Op. cit.*

<sup>6</sup> *Op. cit.*

<sup>7</sup> *Ann. de l'Inst. Past.*, 1902, 16, p. 127.

<sup>8</sup> *Berl. klin. Wchnschr.*, 1902, 5, p. 870.

<sup>9</sup> *Jour. Med. Res.*, 1903, 10, p. 255.

<sup>10</sup> *Centrbl. f. Bakt.*, 1911, 57, p. 577.

<sup>11</sup> *University of California Publications*, 1912, 2, p. 73.

Those tubes in which there was but a moderate or beginning hemolysis were compared.

1. *The amount of complement in paraffin plasma.*—An aspirator with a large needle was filled with liquid paraffin and the paraffin partially expelled. The needle was then inserted into the heart of a guinea-pig and about five cubic centimeters of blood aspirated. The blood was immediately centrifuged in a centrifuge tube coated with liquid paraffin and containing a small amount of paraffin in the bottom. The plasma was then pipetted off and placed in a small test tube. The plasma coagulated at once. A part of the serum was taken from the fibrin and one-half of it used as complement in mixtures which had been made up and were in readiness excepting for the addition of complement. The other half of the serum was incubated for one-half hour as was also the serum fibrin mixture. Two sets of tubes were then arranged similar to the first set, excepting that in the one incubated serum was used as complement, and in the other serum incubated with fibrin. After incubation for two hours all three sets of tubes were read and put on ice until morning and then read again. As is shown in the following table, the fractions of serum treated in the different ways all contained the same amounts of complement.

TABLE I.  
HEMOLYSIS WITH SERUM TREATED IN VARIOUS WAYS.

Treatment of Serum	Per Cent of Hemolysis with Varying Amounts of Serum Diluted 1:100				
	0.05 C.C.	0.1 C.C.	0.2 C.C.	0.3 C.C.	0.4 C.C.
Serum removed from plasma at once.....	0	5	10	25	40
Serum incubated with fibrin one-half hour.....	0	5	10	20	40
Serum incubated alone one-half hour.....	0	5	10	20	50

The difference in the results of this experiment and in those of Gurd<sup>1</sup> may possibly be explained by the fact that ether was used in Gurd's experiments, although an incubation for a short time would serve to get rid of at least part of the ether, yet Guggenheimer<sup>2</sup> has shown that ether has an inhibiting effect on the action of complement.

<sup>1</sup>Op. cit.

<sup>2</sup>Ztschr. f. Immunol., 1911, 11, p. 30.

2. *The influence of the blood-clot and its components upon the complement content of the serum left in contact with it.*—A part of the serum from a few cubic centimeters of guinea-pig blood was obtained by centrifugation immediately after removal and placed on ice. The remainder of the serum was left with the clot in the ice-box for 16 hours. The two parts of the serum were then compared as to the complement content in the way described above in experiment 1. The result was as follows:

TABLE 2.  
HEMOLYSIS WITH SERUM SEPARATED AT ONCE AND LEFT WITH CLOT.

Serum	Per Cent Hemolysis with Varying Amounts of Serum Diluted 1:100		
	0.2 c.c.	0.4 c.c.	0.6 c.c.
Left with clot 16 hours.....	30	60	90
Separated at once.....	40	70	90

There is no difference in the complement content of the two different fractions of the serum which may not be explained by error.

On the supposition that the complement content of serum might be increased by contact with one or more of the components of the clot the following experiment was made. Blood was obtained as in the first experiment and centrifuged in liquid paraffin. As blood so treated separates into a layer of erythrocytes at the bottom, a layer of leukocytes covering this, and above this the plasma, it was possible to obtain a quantity of erythrocytes, leukocytes, and fibrin. Serum left in contact with each of these for 16 hours in the ice-box was compared with serum removed at once.

Table 3 shows that in experiments 1 and 2 there is a diminution of complement content in serum left in contact with white cells and fibrin, whereas in experiment 3 the serum left in contact with the leukocytes is strongest in complement; in experiment 4 the highest complement content is found in that serum which was left in contact with the fibrin, while in experiment 6 the highest content is in the serum incubated with erythrocytes.

It is very unlikely that any of the differences in the tables are

TABLE 3.  
HEMOLYSIS WITH SERUM TREATED VARIOUSLY. THE FIGURES GIVE THE PERCENTAGE OF HEMOLYSIS WITH VARYING AMOUNTS OF SERUM DILUTED 1:100.

Treatment of Serum	Experiment 1 (Serum Incubated with Blood Elements, etc., for 16 Hours)			Experiment 2 (Serum Incubated with Blood Elements, etc., for 16 Hours)			Experiment 3 (Serum Incubated with Blood Elements, etc., for 16 Hours)			Experiment 4 (Serum Incubated Two Hours with Blood Elements, etc.)						Experiment 5 (Serum as in Experiment 4)		
	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	1.0 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Serum removed at once.....	40	80	90	10	20	25	0	10	20	25	10	15	25	30	50	15	20	25
Serum left with clot.....	40	80	90	10	25	30	0	10	20	25	10	15	25	30	50	15	20	25
Serum left with fibrin.....	40	80	90	10	25	30	0	10	25	35	10	20	30	40	60	15	20	25
Serum left with fibrin and leukocytes.....	20	60	80	10	20	25	0	10	20	25	10	15	25	30	50	15	20	25
Serum left with leukocytes.....	40	90	90	10	20	25	0	10	25	40	10	15	25	30	50	15	20	25
Serum left with leukocytes.....	55	90	90	10	25	30	0	10	20	25	10	20	30	35	50	15	25	30
Mixture of equal parts of all sera	40	90	90	10	20	25	0	10	20	25	10	15	25	30	50	15	20	25

outside of the limits of unavoidable error. The experiments therefore fail to indicate any connection between complement formation and the blood-clot or its components. Much of the work which has been taken to indicate that leukocytes are the source of complement has been done on bacteriolytic complement. Bacteriolytic experiments are, as compared with hemolytic, less accurate, and although the possibility of the difference in result being due to a difference in the place of formation of the two complements cannot be denied, the more likely explanation of the difference in the result is in the inaccuracy of bacteriolytic work.

3. *Comparison of the complement content of leukocytic exudates with that of the blood serum.*—The independence of complement content with respect to the number of leukocytes or leukocytic disintegration is shown by a comparison of the amount of complement of fluids rich and poor in leukocytes with that of the serum.

A dog was injected intrapleurally with aleuronat suspension and the following fluids compared: (1) that from the centrifugated leukocytic exudate; (2) the fluid obtained from the centrifugated leukocytes by pressure; (3) the cerebrospinal fluid almost free from leukocytes; (4) the blood serum. It required one and one-half times as much exudate fluid to produce any given percentage of hemolysis as was the case with serum, three times as much leukocytic extract, and four times as much cerebrospinal fluid. The blood serum of the animal might much more reasonably be considered the source of the complement in the various fluids examined than the leukocytes.

Inasmuch as no indication that the leukocytes formed complement could be found, an attempt was made to obtain complement from other parts of the body as follows: A dog was etherized and the femoral vein isolated on one side and the artery on the other. A cannula connected with a reservoir of normal salt solution was inserted into the vein and salt solution allowed to flow in as the dog was bled from the opposite femoral artery. After the liquid from the artery became colorless the various organs were removed from the body and where possible, as in the liver, spleen, and kidney, perfused again with salt solution. Fluids were obtained from the organs by simple pressure, by grinding with salt solution with and

without sand, and by extraction with ether and alcohol. The experiment was repeated a number of times with guinea-pigs and in no instance was a fluid obtained from any of the organs which was capable of acting as complement. In the case of the lipid extracts some hemolysis with the extract alone was obtainable, but the addition of amboceptor inhibited this hemolysis. It is of especial interest to note that good hemolysis was obtainable with extracts of the stomach mucosa, pancreas, and mucosa of the small intestine. Amboceptor, however, instead of increasing the hemolytic power acted as might be expected as an antiferment. The reaction of these extracts was varied, but in no case was a complement action obtainable. Reference will be made to this hemolysis by proteolytic ferments later. Although it was impossible to obtain from any of the organs a fluid or mixture of fluids which would act as complement, these experiments cannot be taken as indication that none of the organs examined are concerned in complement formation. Accordingly a series of experiments was carried out in which the complement content of the blood of dogs was estimated and then in different cases different organs removed and the effect on the complement content of the blood of this removal noted from day to day until the animal died or until a constant complement content was found day after day. In these complement estimations, the same technic was used as was described in comparison of serum treated in different ways. In this case, however, the serum of guinea-pigs of from 200-300 gms. weight was used as a standard for comparison, as it has been shown by many workers and by a few preliminary experiments that the serum of such guinea-pigs is remarkably constant in complement content. The serum of normal dogs under the conditions of the experiments usually contained about one-sixth the complement activity of normal guinea-pig serum. That is, it took about six times as much dog serum as guinea-pig serum to produce a trace of hemolysis.

Inasmuch as dog blood sometimes contains amboceptor for sheep blood, it was necessary to run controls of the action of the dogs' serum alone without the addition of artificial (rabbit) amboceptor. Wherever the dogs' serum was of itself hemolytic for sheep blood the animals were discarded.



*Removal of the pancreas.*—The removal of enough of the pancreas to produce a considerable excretion of sugar in the urine was accomplished in sixteen dogs, of these five developed localized infections so that the experiments were worthless. Of the remaining 11 dogs an acidosis was demonstrated in only six while all had a considerable excretion of sugar in the urine and died after a period of from a few days to two weeks.

Of those animals in which an acidosis was present and sugar excreted, the following may be given as an example of the result on the complement content of the blood serum:

Dog 131. Complement content of the serum March 1, one-fifth that of guinea-pig serum. Three hours after pancreatectomy, one-third; two days later, one-fifth; fourth day, one-seventh; 13th day, one-fifth; dead on the 14th day.

Of these animals showing a constant excretion of sugar, but in which no acidosis was demonstrated, the following will serve as an example:

Dog 96. Complement November 22, one-fifth that of guinea-pig serum; about 24 hours after the operation, November 23, one-sixth; November 24, one-fifth; November 25, one-fifth; November 27, two-fifths; December 1, 10 days after the operation, one-third. The dog died on the 11th day.

As an example of the effect of removal of the pancreas and localized peritonitis the following will serve as an example:

Dog 137. April 1, complement one-fifth that of guinea-pig serum; 24 hours after pancreatectomy, one-fifth; April 6, five days after the operation, no complement could be demonstrated. The dog died soon after the last sample of blood was obtained. These experiments indicate that removal of the pancreas in itself has but little effect upon the complement.

*Removal of pancreas and duodenum.*—Of eight dogs in which the pancreas and duodenum were removed only one showed a change in the complement of the blood. Complement estimations in this dog were as follows:

Dog 104. Before operating, complement one-fifth that of guinea-pig serum. First day after operation, one-half; on succeeding days as follows: one-sixth, one-fifth, one-seventh, one-seventh, one-seventh, one-tenth, one-seventh, one-seventh, one-fifth, one-seventh, one-tenth. On the 14th and 15th days no complement was found and on the 16th day the dog died. No infection was found on postmortem examination.

As an example of the remaining seven dogs which died without change in the complement content of the blood may be given the following:

Dog 108. By mistake no sample before operation was obtained. The first day following operation the complement was one-fifth that of guinea-pig serum. On succeeding days it was two-fifths, one-third, one-tenth, one-third, one-fourth, one-fourth, one-fourth, one-eighth, one-third, one-fifth, one-third. The dog died on the 13th day.

*Removal of spleen and pancreas.*—Two dogs showed much the same result. Only one will be given in detail:

Dog 105. Before operation, complement one-fourth that of guinea-pig serum. On the days succeeding operation, one-fifth, one-third, one-half, one-tenth, one-tenth, one-tenth, one-seventh, one-tenth, one-third, one-third, one-seventh. The dog died on the 12th day.

*Removal of adrenals.*—On account of the difficulty in removal of the adrenals only three animals were obtained in which the results can be used. They lived three, four, and 11 hours after operation. In no case was a diminution of complement found. In the dog which lived 11 hours the following estimations were made. Just before operation, complement one-third. Immediately after operation, one-fourth; one hour later, one-fourth; two and one-half hours later, one-fourth; three and one-half hours later, one-third; four and one-half hours later, one-third; 10½ hours later, one-third.

*Removal of small intestine.*—In each of four dogs approximately six feet of intestine were removed. None of these dogs showed a diminution in complement. One dog lived nearly two months and the complement varied from one-fifth at the beginning to two-fifths a few days before death.

*Gastrectomy.*—It was possible to remove only the pyloric end of the stomach with but a small part of the cardiac end. In the one dog observed the complement was as follows:

Dog 111. Before operation, complement was one-tenth that of guinea-pig serum. First day, one-tenth; second day, one-tenth; third, two-fifths; fourth, two-fifths; sixth, one-fifth; seventh, one-eighth; eighth, one-half; ninth, three-fifths; 10th, one-fifth; 12th, one-tenth; 13th, one-fifth; 14th, one-tenth; 15th, one-fifth; 16th, one-sixth; 18th, one-sixth; 22d, one-sixth; 60th, one-sixth. The dog was examined after killing with chloroform and the organs found normal.

*Thyroidectomy.*—The thyroid was removed in three dogs. One of these was kept alive for over a month by feeding calcium lactate, a fourth dog was phloridzinized after removal of the thyroid. The only dog showing a diminution of complement was the phloridzinized dog and in it the complement rose to normal before death. The complement estimations were as follows: Two days following thyroidectomy, the complement was one-sixth; five days after operation, one-sixth. Phloridzin was then begun and two days later complement was one-tenth. On the following day just before the dog was killed the complement was one-fifth or about normal.

*Kidney.*—No nephrectomies were made but two dogs were injected subcutaneously with bichlorid of mercury. Doses of

one-half and two-tenths gram respectively were given. The first dog died on the following day. The dog receiving two-tenths gm. lived about 55 hours. In neither dog was the complement affected. In the second dog typical bichlorid kidneys were found which on microscopic examination showed extensive destruction of the epithelium and calcium deposit. There was an extensive necrosis of the large intestine with sloughing. The complement estimations were as follows: before the injection, one-sixth; 24 hours later, one-sixth; 48 hours later, one-third. It will be seen that the complement was not diminished by mercuric chlorid poisoning with severe damage to the kidney and colon.

*Liver.*—On account of the difficulty of removing even a part of the liver, the use of poisons which particularly affect the liver was resorted to. Richards and Howland<sup>1</sup> have shown that if dogs are given chloroform by inhalation for two hours or more, severe damage (usually extensive necrosis) is done to the liver. Accordingly chloroform was given to seven dogs for periods ranging from two to three hours; from  $\frac{3}{4}$ –2 oz. were given. In two of the dogs the chloroform administration was repeated. In all of these dogs a marked fall in the complement content of the blood was noted. This fall in complement was synchronous with the change in liver, determined by microscopic examination and roughly parallel to the extent of the liver necrosis. An example of complement estimations with chloroform is as follows:

Dog 170. Given chloroform for two hours. Complement before chloroform, one-sixth that of guinea-pig serum. Two hours after administration of chloroform, one-eighth, 22 hours after, one-twelfth; 46 hours after, one-forty-fifth. The dog died a few hours after the last estimation.

In order to be sure that the apparent diminution in complement was not due to the presence of anticomplementary substances in the blood, the following experiment was made. A dog was given chloroform for two hours and by repeated estimations a fall of complement from one-sixth before the chloroform to one-twentieth 96 hours after was ascertained. At this time a quantity of serum was obtained and heated to 56° C. for one-half hour. The minimum amount of normal dog serum required to cause

<sup>1</sup> *Jour. Exper. Med.*, 1900, 11, p. 344.

complete hemolysis with 0.01 c.c. of amboceptor was then found. Then the amounts of heated normal and heated abnormal serum required to inhibit the action of the minimum dose of normal dog serum required to cause complete hemolysis was determined. There was no increase in the inhibiting power of the serum of the chloroformed dogs. An attempt was made to increase the activity of the serum of the chloroformed dog by variation of the reaction of the hemolytic mixtures, using constant quantities of different concentrations of acids and alkalies. In this way concentrations of N/40,000, N/30,000, N/20,000, N/10,000, N/5,000, N/3,500, N/2,000 NaOH and the same strengths of HCl were used. In no case was the complement action increased.

It was suggested by Dr. Wells<sup>1</sup> that the action of hydrazine to be tried in this connection as he has shown that<sup>2</sup> hydrazine is a poison which acts in a peculiarly specific way upon the liver cells.

Dog 150 was given 1.2 gms. of hydrazine sulphate (0.1 gm. per kgm. body weight) subcutaneously. The complement estimations were as follows. Before injection of hydrazine, complement was one-fifth; 24 hours later, one-eighth; 48 hours later, one-tenth; 72 hours later, one-fortieth. The dog died a few hours after the last sample was taken. Extensive necrosis was found microscopically in the liver.

It has been shown by Whipple and Hurwitz<sup>3</sup> that in chloroformed animals a drop in fibrinogen occurs which is proportional to the amount of liver necrosis. Inasmuch as both the fibrinogen and the so-called middle-piece of the complement belong to the globulin fraction of the serum, it was thought desirable to find out whether or not alterations in the middle-piece caused the drop in complement. Accordingly the serum from dog 170 was separated into middle-piece and end-piece by the hydrochloric acid method described by Marks<sup>4</sup> and solutions of end-piece and middle-piece representing each dilution of the original concentration in the serum of 1:10, compared with similar solutions of the middle-piece and end-piece of normal serum. It will be seen from the following table (4) that the middle-piece of the complement-poor serum is equal to the normal middle-piece in concentration and that the drop in complement is due to a change in the end-piece or albumen fraction and not the globulin fraction as was suspected.

<sup>1</sup> *Ibid.*, 1908, 10, p. 437.

<sup>2</sup> *Ibid.*, 1911, 3, p. 136.

<sup>3</sup> *Ztschr. f. Immunol.*, 1910, 8, p. 508.

TABLE 4.

THE ACTION OF COMPLEMENT-FRACTIONS ON HEMOLYSIS.  
 Normal end-piece alone hemolyzes with 0.5 c.c., a trace with 0.4 c.c., none with 0.3 c.c.  
 Complement poor end-piece does not hemolyze.

NORMAL END-PIECE, 0.2 C.C.		NORMAL END-PIECE, 0.3 C.C.	
Liver End-Piece	Normal End-Piece	Liver Mid-Piece	Normal Mid-Piece
0.1 c.c. no hemolysis	0.1 c.c. complete hemolysis	0.1 c.c. complete hemolysis	0.1 c.c. moderate hemolysis
0.2 c.c. no hemolysis	0.2 c.c. complete hemolysis	0.2 c.c. complete hemolysis	0.2 c.c. moderate hemolysis
0.3 c.c. trace	0.3 c.c. complete hemolysis	0.3 c.c. complete hemolysis	0.3 c.c. moderate hemolysis
0.4 c.c. moderate hemolysis	0.4 c.c. complete hemolysis	0.4 c.c. complete hemolysis	0.4 c.c. moderate hemolysis
0.5 c.c. complete hemolysis	0.5 c.c. complete hemolysis	0.5 c.c. complete hemolysis	0.5 c.c. moderate hemolysis

In 1900, Ehrlich and Morgenroth<sup>1</sup> noted that guinea-pig serum loses its hemolytic power for rabbit corpuscles when the animal is poisoned with phosphorus. They were able to restore this power by adding normal guinea-pig serum and concluded that it was the complement that was affected. We have three substances which cause a destruction particularly of the liver tissue and at the same time a diminution of the complement of the blood. This parallelism between complement diminution and liver destruction suggests that either the complement is formed in the liver or that destruction of the liver cells inhibits the formation of complement somewhere else. Inasmuch as these experiments failed to demonstrate any other place of formation, the indications are that it is actually formed in the liver.

#### THE ACTION OF COMPLEMENT.

It has been a much disputed idea that the complement of the blood is a ferment. In the course of a discussion concerning the ferment nature of complement, Lieberman and Fenyvessy call attention to the fact that none of those who maintain that complement is a ferment advance any ideas as to what chemical processes are concerned in complement action. This investigation has been concerned with (I) the ferment nature of complement; (II) the nature of the hemolytic action of complement and immune body.

I. *The ferment nature of complement.*—The arguments which

<sup>1</sup> *Berl. klin. Wchnschr.*, 1900, 37, p. 683.

have been advanced in favor of the ferment nature of complement are as follows:

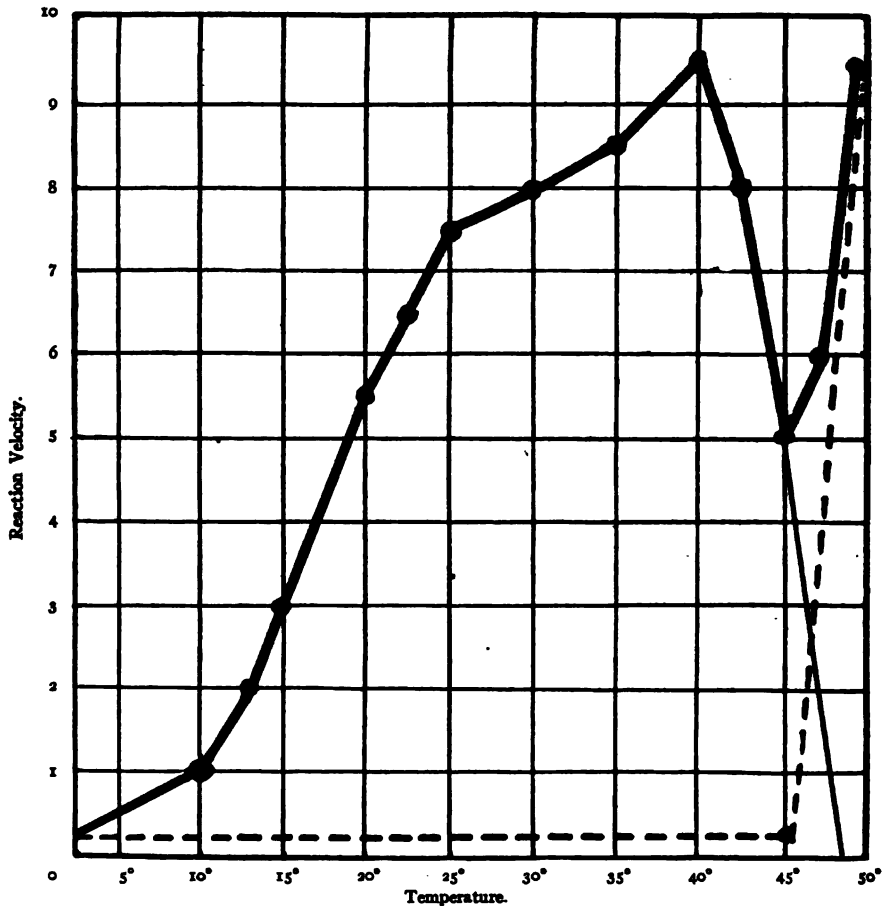
1. Complement and immune body do not necessarily act together in definite proportions but an increase in the amount of the one may act as a substitute for a diminution of the other.
2. The reaction velocity of hemolysis depends rather on the concentration than on the absolute amount of complement.
3. Complement is not used up during hemolysis.
4. The action of complement is similar to that of ferment in that it is easily influenced by variations in reactions and salt concentration.
5. The reaction velocity of hemolysis is greatest during the beginning of the process and progressively becomes slower. If this change in reaction velocity is represented by a curve, the curve is very similar in its course to that representing the reaction velocity of trypsin.

It has been shown that instead of the steady increase in velocity of chemical action with increase in temperature, of ordinary chemical reactions, ferment activity shows a marked rise in activity up to a certain optimum and then as the temperature approaches that of the point of destruction of the ferment a sharp fall occurs. Human serum in constant quantity and concentration was allowed to act upon rabbit corpuscles in constant quantity for one hour at different temperatures. The amount of spontaneous hemolysis was estimated by mixtures of heated ( $56^{\circ}\text{C}$ . for one-half hour) human serum and corpuscles. The result is expressed in the following curve which resembles closely these representing typical ferment action.

II. *The nature of the chemical processes concerned in hemolysis.*—The close association of complement with proteolytic processes has already been referred to. In the first part of this paper the hemolytic action of extracts of stomach mucosa was noted. The assumption of a proteolysis of the stroma of erythrocytes would readily explain the phenomenon of hemolysis, for with even partial splitting of the complex protein substance of the stroma there would result an increased permeability which would permit of the escape of hemoglobin.

The following experiments were carried out with a view of obtaining direct evidence that proteolysis occurs during hemolysis. A preparation of the stroma of sheep erythrocytes was made accord-

CURVE SHOWING THE INFLUENCE OF TEMPERATURE ON THE REACTION VELOCITY OF COMPLEMENT.



Dotted line indicates action of serum heated to 56° C. for one-half hour.

Heavy continuous line indicates action of normal serum.

At 45° C. the two curves coincide. Fine continuous line indicates reaction velocity of complement action, computed by subtracting percentage of "spontaneous" hemolysis, obtained with heated serum, from percentage caused by unheated serum.

10 per cent hemolysis = a velocity of 1.

ing to Woolridges' method. Two-tenths gram of the dried stroma was powdered in a mortar and suspended in 20 c.c. of normal salt

solution. This suspension was divided into two portions of 10 c.c. each. These two portions were labeled No. I and No. II. To part I were added 0.4 c.c. of amboceptor and one cubic centimeter of complement (guinea-pig serum.) To part II were added 0.4 c.c. of amboceptor and one cubic centimeter of complement which had been heated to 56° C. for one-half hour. The different components were all cooled to nearly freezing point before mixing and as soon as the mixtures were made they were each divided into two equal parts, one of which was immediately centrifuged and five cubic centimeters of the supernatant fluid titrated by the Henriques and Sorenson method for amino-acids. The other half was incubated for two and one-half hours and then treated the same as the first half. The results were as follows:

Mixture I. Ten c.c. of sheep blood stroma suspension, 0.4 c.c. of amboceptor and one cubic centimeter of complement. Formol titration for amino-acids of 5 c.c. of supernatant fluid before incubation, 0.30 c.c. of N/10 NaOH. After incubation, 0.50 c.c. of N/10NaOH. The difference represents the amount of amino-acid formed during the incubation of 0.20 c.c. of N/10 NaOH.

Mixture II. Ten c.c. of sheep blood stroma suspension, 0.4 c.c. of amboceptor, one cubic centimeter of complement. Formol titration of five cubic centimeters of supernatant fluid for amino-acids before incubation, 0.30 c.c. of N/10 normal NaOH. After incubation 0.40 c.c. of N/10 NaOH. The difference represents the amount of amino-acid formed during the incubation of 10 c.c. of N/10 NaOH.

This experiment was repeated a number of times and although the results were similar and tended to show an increased amount of amino-acid in mixtures of stroma, amboceptor, and complement, still the differences were so small that it is questionable if they were outside of the limits of unavoidable error. Accordingly unchanged blood was used in larger quantities and the disturbance of color reactions due to hemoglobin avoided by removing the coagulable albumen in the mixture before estimating the amino-acids. An example of one of these experiments is the following:

Three mixtures were made. No. I contained 15 c.c. of a 100 per cent suspension of washed sheep erythrocytes, 3.5 c.c. of amboceptor (antisheep rabbit serum), and six cubic centimeters of complement (guinea-pig serum). No. II, 15 c.c. of the same blood suspension as No. I and 9.5 c.c. of normal salt solution. No. III, 15 c.c. of normal salt solution, 3.5 c.c. of amboceptor and five cubic centimeters of complement. The mixtures were cooled and 10 c.c. of each of the mixtures treated as follows to remove the coagulable protein. The mixture was heated to 80° C. in a water bath, one per cent acetic acid was added until the first appearance of an acid reaction to



litmus paper and the mixture then boiled for a few minutes and filtered. The coagulable protein was washed with enough water to finally make the volume of the filtrate 100 c.c. and the amino-acids estimated as before. The remaining parts of the three mixtures were incubated under toluol for 16 hours and at the end of that time the amount of amino-acids in the uncoagulable protein of 10 c.c. of each estimated as above. The table shows that a marked increase in the amino-acids of the hemolytic mixtures occurred upon incubation.

MIXTURES	CURIC CENTIMETER OF 1/20 NaOH IN FORMOL TITRATION	
	Before Incubation	After Incubation
Mixture I. 15 c.c. of blood suspension 3.5 c.c. of amboceptor, 6.0 c.c. of complement.....	0.6 c.c.	1.25 c.c.
Mixture II. 15 c.c. of blood suspension, 0.5 c.c. of salt solution.....	0.4 c.c.	0.4 c.c.
Mixture III. 15 c.c. of salt solution, 3.5 c.c. of amboceptor, 6.0 c.c. of complement.....	0.4 c.c.	0.4 c.c.

Repetition of this experiment gave constantly an increase in amino-acids in the corpuscle amboceptor complement mixtures during incubation, thus showing that proteolysis occurred during the process of hemolysis. Although the demonstration of the occurrence of proteolysis during hemolysis is not proof that it is the cause of the phenomenon, still it suggests rather strongly that such is the case.

#### CONCLUSIONS.

The results of this investigation would indicate that hemolytic complement is a proteolytic ferment which is either formed in the liver or is dependent upon liver activity for its presence in the blood.

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## THE RELATION OF CALCIUM TO ANAPHYLAXIS.\*

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In continuing the investigations regarding the etiology of parturient paresis (milk fever), it occurred to the writers that a study of the effect of the intraperitoneal injection of soluble calcium salts on the normal guinea-pig, and whether these salts exert any antianaphylactic action, would be of great interest. So far as we are able to ascertain from an examination of the literature at present available, very little is known concerning the action of intraperitoneal injection of soluble calcium salts upon the lower animals.

When injected directly into the blood, calcium salts seem to be poisonous, their action resembling that of digitalis in some respects. Cushing<sup>†</sup> states that the lime salts penetrate the tissues with difficulty, and are largely excreted through the intestinal epithelium. Soluble calcium salts precipitate proteins, and when perfused through certain tissues, lime salts are retained by a process of selective absorption. It is also known that calcium shows a remarkable antagonism to certain other metals in their action on

\* Received for publication January 26, 1913.

† *Pharmacology and Therapeutics*, 1911, p. 571.

the lower animals and on isolated organs and tissues. Thus the action of potassium on the frog's heart is antagonized by calcium, and as pointed out by Meltzer and Auer,<sup>1</sup> calcium shows a remarkably antagonistic effect toward magnesium. Indeed the more thoroughly the whole subject of calcium in its relation to the animal and plant economy has been studied, the more clearly has it been recognized how essential the calcium salts are to the activity of certain phenomena and ferments.

From his studies on anaphylaxis, Besredka<sup>2</sup> arrived at the conclusion that calcium chlorid is *par excellence* the great anti-anaphylactic. He states that calcium chlorid, when injected the day before the administration of the second dose of serum, prevents the onset of anaphylaxis, when the sensitized guinea-pigs receive the second dose, 0.25 c.c. of serum, by intraperitoneal injection. In this communication he reserved the right to continue his investigation of the arrest of anaphylactic shock, but since this time (1907) we have been unable to find anything further by this author, regarding the use of calcium chlorid as an antianaphylactic. It is of interest to note in this connection that Netter<sup>3</sup> has shown that in children the eruption following the injection of serum is greatly reduced by the administration of calcium chlorid on the day of the injection and on the two following days. Rosenau and Anderson<sup>4</sup> failed to confirm Besredka's observation regarding the protective action of calcium chlorid against anaphylactic shock. According to these last-named observers, no favorable influence upon the anaphylactic state was obtained by the subcutaneous injection of calcium chlorid, the day preceding the second dose of serum. They also found that the toxic principle of horse serum is not altered in any way by calcium chlorid nor did the administration of calcium chlorid to guinea-pigs by mouth, for several days before the second injection, influence the anaphylaxis.

That the five per cent solution of calcium chlorid, used by Rosenau and Anderson, is very irritating when used subcutaneously, and therefore unsuitable for the purpose of demonstrating the

<sup>1</sup> *Am. Jour. of Physiol.*, 1908, 21, p. 400.

<sup>2</sup> *Compt. rend. Soc. de biol.*, 1907, 62, p. 1053.

<sup>3</sup> *Ibid.*, 1906, 60, p. 279.

<sup>4</sup> U.S. Public Health and Marine Hospital Service *Bull.* 50, 1908.

effect of calcium upon anaphylaxis, is clearly shown by the following experiment.

Four normal adult guinea-pigs received, by subcutaneous injection into the anterior abdominal wall, three cubic centimeters of a five per cent sterile solution of calcium chlorid. Two of these pigs died within 48 hours of acute peritonitis, accompanied by considerable inflammation at the site of the injection. The remaining two pigs developed large abscesses in the anterior abdominal wall. These abscesses ruptured at the end of seven days, leaving rapidly ulcerating sores which opened into the general peritoneal cavity, resulting in the death of the animals, 16 days after the injection of the calcium.

Two<sup>1</sup> of the writers have demonstrated that a 3.25 per cent solution of calcium lactate produced no harmful effects, other than causing abortion, when injected into the peritoneal cavity of the female guinea-pig. It was therefore decided to use this solution in the present investigations. At the beginning of these experiments with calcium lactate, it was observed that while a 3.25 per cent solution of calcium lactate was practically harmless for female guinea-pigs, yet it was very poisonous for male pigs. We have records of eight normal male guinea-pigs which received, intraperitoneally, nine cubic centimeters of the above solution. Of these eight pigs, five died within 24 hours—a mortality of over 62 per cent. On postmortem examination none of the five pigs presented any evidence of injury, nor gross pathological lesions, except a marked congestion of the kidneys. On microscopical examination of the organs of these pigs, one observed in the liver an extensive cloudy swelling of the cells, with greatly distended capillaries of the centrilobular veins; in places these capillaries closely resembled small interstitial hemorrhages; in the kidney there was also cloudy swelling of the epithelium, of the convoluted tubules; in many places the epithelial cells had entirely disappeared; and there were many small and a few large interstitial hemorrhages; in the adrenals, extreme engorgement of the veins and capillaries and minute interstitial hemorrhages.

Smaller doses of the calcium lactate were now used with much better results. Thirteen guinea-pigs, of which eight were males

<sup>1</sup> Kastle and Healy, *Jour. Infect. Dis.*, 1911, 10, p. 378.

and five females, received, intraperitoneally, from 1 c.c. to 4 c.c. of a 3.25 per cent solution of calcium lactate, and suffered no bad after effects.

*Experiment 1.*—Two normal male guinea-pigs received, intraperitoneally, five cubic centimeters of fresh whole milk. Five weeks later they received, in a similar manner, 10 c.c. of the calcium solution. One of these pigs died within 24 hours following the calcium injection. The other pig stood the calcium injection well, and at the end of 24 hours received, intraperitoneally, four cubic centimeters of fresh skimmed milk. With the exception of a little restlessness, this pig showed no symptoms following the second injection of milk, and remained quiet with no inclination to eat for about five hours, after which it was apparently normal and, remaining so, was returned to the piggery in good health a month later.

*Experiment 2.*—Ten normal guinea-pigs received, intraperitoneally, five cubic centimeters of fresh horse serum. Twenty-two days later five of these pigs received, intraperitoneally, 10 c.c. of a 3.25 per cent calcium lactate solution. Three of these pigs died within 24 hours following the calcium injection. On the twenty-third day following the initial dose of horse serum, the surviving seven pigs received, intraperitoneally, a second dose of five cubic centimeters of horse serum. Unfortunately the horse serum which was used in this experiment was not very toxic, so that none of the pigs died following the second dose. Those pigs, however, which had received no calcium, showed, with one exception, distinct symptoms of anaphylaxis, such as restlessness, rubbing of the nose and face, followed by prostration within one hour. One pig showed no symptoms whatever, and three hours after the second dose, all were apparently normal. The two pigs which survived the calcium lactate, upon receiving the second dose of horse serum, were restless with rubbing of the nose and face for a few moments only, after which they showed no symptoms whatever, and at the end of three hours were apparently normal.

*Experiment 3.*—Five young normal male guinea-pigs received, intraperitoneally, a dose which varied from 1 c.c. to 8 c.c. of the above calcium lactate solution. The two pigs receiving 6 c.c. and 8 c.c., respectively, died within 24 hours following the calcium injection. On the following day each of the three surviving pigs received, intraperitoneally, five cubic centimeters of a solution containing equal parts of egg-white and distilled water. At the end of 18 days each of these pigs received, in the usual manner, five cubic centimeters of a solution containing equal parts of egg-white and distilled water. Following this second dose of egg-white, two of these pigs had violent convulsions, while the third had violent respiratory spasms, and all died within 25 minutes. On postmortem examination of these pigs, no evidence of injury nor gross pathological lesions were found.

*Experiment 4.*—Five somewhat larger male guinea-pigs received, intraperitoneally, five cubic centimeters of the above solution of egg-white. They all stood the injection well. At the end of 18 days, four of these pigs received, in the usual manner, 1 c.c., 2 c.c., 3 c.c., and 4 c.c. of the calcium lactate solution. The fifth pig, used as a control, received no calcium. On the following day all five pigs received, intraperitoneally, five cubic centimeters of the egg-white solution. Three of the four pigs which had received the calcium had distinct symptoms of anaphylaxis, consisting of scratching of the nose, rapid respirations, accompanied in some cases by a clicking sound, and restlessness, but all recovered. The fourth pig injected with

calcium had violent convulsions and died in 21 minutes. The control pig also had violent convulsions and died in 23 minutes. On postmortem examination of these two pigs, no evidence of injury nor gross pathological lesions were found.

*Experiment 5.*—Six normal female guinea-pigs received, intraperitoneally, five cubic centimeters of a solution containing one part of egg-white and two parts of distilled water. They all stood this injection well. The following day each of two of these pigs received, intraperitoneally, two cubic centimeters of the calcium lactate solution. At the end of six days each of two other pigs received, in the same manner, a similar dose of calcium lactate, and finally, at the end of 15 days, one of the two remaining pigs received a similar dose of calcium. At the end of 16 days all six pigs were in good condition, and all received, intraperitoneally, five cubic centimeters of the above solution of egg-white. Following this second dose the pig which had received no calcium died in convulsions in five and one-half minutes; the two pigs which had received the calcium at the end of 24 hours, following the first dose of egg-white, died in convulsions in 33 and 56 minutes, respectively; one of the two pigs which had received the calcium at the end of six days died in convulsions in 19 minutes, while the other of these two pigs showed some mild symptoms, and recovered; finally, the pig receiving the calcium 24 hours before the second dose of egg-white also presented mild symptoms of anaphylaxis, and also recovered.

*Experiment 6.*—Three male guinea-pigs, which had survived from the fourth experiment and which had been protected against the second dose of egg-white through the action of calcium, received, intraperitoneally, five cubic centimeters of fresh undiluted cat serum. Fourteen weeks later two of these pigs received, in the usual manner, two cubic centimeters of the calcium solution. They both stood the injection well, and on the following day they all received 2.5 c.c. of fresh cat serum. The pig which had received no calcium had mild convulsions, and died very quietly in three and one-half hours; of the two pigs which had received calcium, one had slight convulsions and died very quietly in two and one-quarter hours; the other had slight symptoms of anaphylaxis, and recovered.

#### CONCLUSIONS.

As a result of the above experiments the following three conclusions seem inevitable: first, that a five per cent solution of calcium chlorid used subcutaneously is much too irritating to allow of a fair estimate of the value of calcium as an antianaphylactic; second, that an isotonic solution of calcium lactate used intraperitoneally, in large doses of 5-10 c.c., is very poisonous to male guinea-pigs, the mortality being over 62 per cent; third, that an isotonic solution of calcium lactate used intraperitoneally, in small doses of 1-4 c.c., and at the proper time, does protect guinea-pigs from the anaphylactic shock resulting from milk, horse serum, egg-white, and cat serum. In the first experiment, in which milk was used, the fact that only one pig survived the calcium leaves the question somewhat in doubt, although it is

quite reasonable to infer that the calcium saved the second pig. In the second experiment, in which horse serum was used, there is again doubt, due to the fact that the horse serum was not very toxic. In the third, fourth, and fifth experiments, however, there is no room for doubt. In these experiments egg-white was employed as the anaphylactic agent with a mortality of 88.9 per cent, whereas the mortality from the second dose of egg-white, following the injection of an isotonic solution of calcium lactate, was only 20 per cent.

The writers, therefore, are disposed to agree with Besredka that the use of calcium in the proper doses and at the proper time does protect the animal against anaphylactic shock.

These investigations are being continued.

## ISOAGGLUTINATION IN MAN AND LOWER ANIMALS.\*†

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It is of interest that the normal serum of one individual may agglutinate the red blood cells of another of the same species, a phenomenon which Ehrlich named isoagglutination.

Landsteiner<sup>1</sup> first suggested that individuals might be divided into three distinct groups according to this phenomenon:

*Group 1.*—The corpuscles are not agglutinated by sera of the other two groups, while the sera agglutinate the corpuscles of both groups.

*Group 2.*—The corpuscles are agglutinated by the sera of the other two groups while the sera agglutinate the corpuscles of group 3, but not of group 1.

*Group 3.*—The corpuscles are agglutinated by the other two sera, and the sera agglutinate the corpuscles of group 2, but not of group 1.

Jansky<sup>2</sup> and Moss<sup>3</sup> divided individuals into four groups. Moss found isoagglutination in 90 per cent of the individuals tested. His grouping and the percentage in each group are as follows:

*Group 1.*—Ten per cent; serum agglutinates corpuscles of no group; corpuscles agglutinated by serum of groups 2, 3, and 4.

*Group 2.*—Forty per cent; serum agglutinates corpuscles of groups 1 and 3; corpuscles agglutinated by serum of groups 3 and 4.

*Group 3.*—Seven per cent; serum agglutinates corpuscles of groups 1 and 2; corpuscles agglutinated by serum of groups 2 and 4.

*Group 4.*—Forty-three per cent; serum agglutinates corpuscles of groups 1, 2, and 3; corpuscles are not agglutinated by any serum.

There seem to be no autoagglutinins.

Isoagglutination may possibly bring about disastrous results in transfusion of blood, and the selection of a donor should be made with reference to the isoagglutinative grouping. Manifestly the greatest danger lies in transfusion between members of groups 2 and 3. Cases with seemingly unfavorable results under these

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† This work has been aided by the Alumni Fellowship of Rush Medical College.

<sup>1</sup> Landsteiner, *Centralbl. f. Bakt.*, I, Orig., 1900, 27, p. 361; *Wien. klin. Wchschr.*, 1901, 14, p. 1132; Landsteiner and Leiner, *Centralbl. f. Bakt.*, I, Orig., 1905, 38, p. 548; Landsteiner and Reich, *Ibid.*, 1905, 39, p. 712.

<sup>2</sup> Cited by Moss.

<sup>3</sup> *Johns Hopkins Hosp. Bull.*, 1910, 21, p. 63.



conditions have been reported by Schultz,<sup>1</sup> Ottenberg,<sup>2</sup> and Hopkins.<sup>3</sup>

Concerning the nature of the so-called isoagglutinins it has been shown that they are relatively thermostable, and resist heating to 55° C. for 30 minutes. Halban has stated that fetal blood from the cord at birth often contains isoagglutinin, even in cases in which the maternal blood did not contain isoagglutinins. Ottenberg and Epstein<sup>4</sup> and later von Dungern and Hirschfeld conclude that with respect to inheritance isoagglutination appears to follow the Mendelian law. When strong serum is used agglutination may take place almost instantaneously. The isoagglutinative substances are absorbed by the cells which they agglutinate. Hektoen<sup>5</sup> and Moss<sup>6</sup> found that the isoagglutination occurs with approximately the same relative frequency in health as in disease. Hektoen found that the concentration of the isoagglutinins is practically constant from day to day in the same individuals. Gay<sup>7</sup> urged that there is evidence for the belief that isoagglutination of human blood may be due simply to physico-chemical variation of molecular concentration and not dependent on the presence of any agglutinins.

In performing isoagglutinative tests various methods have been used. Practically all observers have used a five per cent suspension of corpuscles and mixed with a small amount of this suspension in small tubes an equal quantity of serum. Ottenberg and Epstein make the tests in capillary pipettes. In making tests on large groups, difficulty was experienced with both methods and a new method<sup>8</sup> was therefore attempted, which proved of great value in carrying out tests for isoagglutination rapidly in large groups.

Three drops of the blood are added to 10 c.c. of a 1 per cent solution of sodium citrate in physiologic salt solution. In this manner approximately a 2 per cent suspension of the blood is prepared, the citrate preventing coagulation. More blood is collected in a centrifuge tube and allowed to clot. With a clean needle the clot is loosened from the side of the tube and the tube centrifuged for a few minutes to obtain an upper layer of absolutely clear serum. This completes the preparation

<sup>1</sup> *Berl. klin. Wchnschr.*, 1910, 47, p. 1407.

<sup>2</sup> *Jour. Exper. Med.*, 1911, 13, p. 425.

<sup>3</sup> *Arch. Int. Med.*, 1910, 6, p. 270.

<sup>4</sup> *Trans. Amer. Assoc. Physicians*, 1909, 24, p. 419.

<sup>5</sup> *Jour. Med. Res.*, 1908, 17, p. 321.

<sup>6</sup> *Trans. New York Path. Soc.*, 1908, 8, p. 117.

<sup>7</sup> *Jour. Infect. Dis.*, 1907, 4, p. 297.

<sup>8</sup> *Jour. Amer. Med. Assoc.*, 1912, 59, p. 793.

of material, with the exception of the plate which is now described. On an ordinary piece of window glass, approximately  $3\frac{1}{2} \times 9$  inches in size, which has been washed clean with water and ether, small circles are made with melted paraffin. If the melted paraffin is drawn up into a medicine dropper, the circles are quickly made with the tip while gentle pressure is exerted on the bulb. Ten circles may be made with one medicine dropper full of melted paraffin. In this manner paraffin cups are made, each of which will hold at least four drops. Assuming that a series of 20 are to be studied, 40 circles may be made on one such plate, four rows of 10 each. Ten such plates are prepared. Then into each cup of set No. 1 are placed two drops of serum 1; in each cup of set No. 2 are placed two drops of serum 2, etc. In each of the 20 cups in set No. 1 are placed next two drops of corpuscles No. 1, etc. If there is still likelihood of clotting, a drop of citrate solution may be added to each mixture. With a narrow glass rod the fluids are mixed thoroughly, the rod being washed in citrate solution and wiped after each mixing. In practically all instances, isoagglutination when present becomes visible macroscopically after half an hour at room temperature; the mixture can be inspected easily under the microscope also.

Two groups of normal individuals were examined and the same definite grouping as has been observed by the various investigators was found. A typical table is given (Table 1).

TABLE 1.  
ISOAGGLUTINATION IN MAN.

Corpuscles	Serum																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
I.....	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
II.....	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
III.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
IV.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
V.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
VI.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
VII.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
VIII.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
IX.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
X.....	o	o	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+
XI.....	o	o	+	+	+	+	+	+	+	+	o	+	+	+	+	+	+	+	+	+
XII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XIV.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XV.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XVI.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XVII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XVIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XIX.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XX.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

As isoagglutination would seem to be a phenomenon of biological importance, a study of the occurrence in various species was undertaken. Hektoen failed to observe isoagglutinins in the serum of rabbits, guinea-pigs, dogs, horses, and cattle, in each instance

using the serum and corpuscles of 10 to 20 different animals. Ottenberg and Friedman,<sup>1</sup> however, found that it did occur in rabbits and in steers. In rabbits it was found that there occurred a distinct grouping which bore no relation to race and color. These groups were as follows:

*Group 1.*—Serum agglutinative toward all cells, cells not agglutinable.

*Group 2.*—Serum agglutinates group 3 cells, cells agglutinated by serum 1.

*Group 3.*—Serum non-agglutinative, cells agglutinated by sera 1 and 2.

*Group 4.*—Serum non-agglutinative, cells not agglutinable.

In steers it was found that a grouping could be made into three sets:

*Group 1.*—Agglutinative but not agglutinable.

*Group 2.*—Agglutinable but not agglutinative.

*Group 3.*—Neither agglutinative nor agglutinable.

More recently Ingebrigsten<sup>2</sup> studied the occurrence of isoagglutination in 40 cats. He found in at least five cases positive interagglutination. In other instances there was some doubt. In 32 no agglutination was present. No grouping was possible.

I have examined several different species including 60 swine, 60 cattle, 40 sheep, 25 rabbits, 20 frogs, and 10 dogs. Tables 2, 3, 4, and 5 of groups of twenty are typical of the results.

TABLE 2.  
ISOAGGLUTINATION IN SHEEP.

Corpuscles	Serum																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
I.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
II.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
III.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
IV.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
V.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
VI.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
VII.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
VIII.....	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
IX.....	o	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
X.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XI.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XIV.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XV.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XVI.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XVII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XVIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XIX.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
XX.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

<sup>1</sup> *Jour. Exper. Med.*, 1911, 13, p. 531.

<sup>2</sup> *München. med. Wchnschr.*, 1912, 59, p. 1475.

In studying isoagglutination in these animals, an endeavor was made to determine whether race or color played any part. It was found that the occurrence of agglutination did not bear any relationship to these factors.

It was found further that isoagglutination in animals is a much less pronounced phenomenon than in man. In many

TABLE 3.  
ISOAGGLUTINATION IN SWINE.

Cor- puscles	Serum																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
I.....	o	o	o	o	o	o	o	o	o	+	o	+	+	+	+	o	o	o	+	o
II.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
III.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
IV.....	+	o	o	o	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
V.....	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
VI.....	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
VII.....	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	o	o	+	o
VIII.....	+	o	o	o	o	o	o	o	o	o	o	+	o	o	+	+	o	o	+	o
IX.....	+	o	o	o	o	+	o	o	o	o	o	+	o	o	+	+	o	o	+	o
X.....	+	o	o	o	o	o	o	o	o	o	o	+	o	o	+	+	o	o	+	o
XI.....	+	o	o	o	o	+	o	o	o	o	o	+	o	o	+	+	o	o	+	o
XII.....	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	+	o
XIII.....	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
XIV.....	+	o	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	o	+	o
XV.....	+	o	o	o	+	o	o	o	o	o	o	o	o	+	o	o	o	o	+	o
XVI.....	+	o	o	o	o	o	o	o	o	o	+	o	o	o	o	o	o	o	+	o
XVII.....	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
XVIII.....	+	o	o	o	+	o	o	+	+	+	+	+	+	+	+	+	o	o	+	+
XIX.....	+	+	+	+	+	o	o	+	+	+	+	+	+	+	+	+	o	o	+	+
XX.....	+	+	+	+	+	o	o	+	+	+	+	+	+	+	+	+	o	o	+	+

TABLE 4.  
ISOAGGLUTINATION IN CATTLE.

Cor- puscles	Serum																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
I.....	o	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
II.....	o	+	o	o	o	o	o	o	o	o	o	o	o	o	+	+	o	+	o	o
III.....	o	+	o	o	o	o	o	o	o	o	o	o	o	o	+	+	o	+	o	o
IV.....	o	+	o	o	o	o	o	o	o	o	o	o	o	+	+	+	o	+	o	o
V.....	+	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	o	+	o	o
VI.....	+	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	o	+	o	o
VII.....	+	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	o	+	o	o
VIII.....	+	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	o	+	o	o
IX.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
X.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XI.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XII.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XIII.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XIV.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XV.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XVI.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XVII.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XVIII.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XIX.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XX.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

TABLE 5.  
ISOAGGLUTINATION IN RABBITS.

Cor- puscles	Serum.																				Species
	1	2	3	4	5	6	6	8	9	10	11	12	13	14	15	16	17	18	19	20	
I.....	o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Tan
II.....	o	o	o	o	o	o	o	o	o	+	+	o	o	o	o	+	o	o	o	o	Grey
III.....	o	o	o	o	o	o	o	o	o	+	+	o	o	o	o	+	o	o	o	o	White
IV.....	o	o	o	o	o	o	o	o	o	+	+	o	o	o	o	+	o	o	o	o	Belgian
V.....	o	o	o	o	o	o	o	o	o	o	+	o	o	o	o	o	o	o	o	o	"
VI.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	"
VII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	o	o	o	"
VIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	Black
IX.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	Grey
X.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	White
XI.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	Tan
XII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	Grey
XIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	and
XIV.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	White
XV.....	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	o	Grey
XVI.....	o	o	o	o	o	o	o	o	o	o	+	o	o	+	o	o	o	o	o	o	White
XVII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	o	o	o	Grey
XVIII.....	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	o	o	o	and
XIX.....	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o	+	o	o	o	o	White
XX.....	o	o	+	o	o	o	o	o	o	o	o	o	o	+	o	+	o	o	o	o	"

instances, only by microscopical examination could it be accurately determined whether agglutination had occurred or not. This was particularly true in the case of frogs and rabbits.

It is obvious from the tables that no division into groups can be consistently made. Just as Ingebrigsten found to be the case in cats, so in dogs, sheep, cattle, swine, and rabbits, isoagglutination occurs but seems to be governed by no definite grouping. In all instances there is indeed a large group which is neither agglutinative nor agglutinable, this being particularly true of sheep and rabbits, but beyond this nothing further can be said.

In the frogs examined, isoagglutination did not occur. All specimens were examined microscopically at various intervals after mixing, but not the slightest trace of agglutination was visible.

With regard to isoagglutination in swine, Reichel<sup>1</sup> states that in intravenous injection of hogs with hog serum, deaths have been observed with all the evidences of intravascular agglutination.

The mechanism of this phenomenon is still in doubt. Various interesting hypotheses have been advanced and but few experiments made in their support. The assumption is usually made of

<sup>1</sup> Personal communication.

various agglutinins and agglutinophilic receptors. Thus Moss advances the following explanation for the grouping in man:

*Group 1.*—Serum contains no agglutinin, corpuscles possess receptors *a*, *b*, and *c*.

*Group 2.*—Serum contains agglutinin A, corpuscles possess receptors *b* and *c*.

*Group 3.*—Serum contains agglutinin B, corpuscles possess receptors *a* and *c*.

*Group 4.*—Serum contains agglutinin C, corpuscles possess no receptors.

It would seem that the only other possibility would be the reverse, agglutinins being substituted for receptors and vice versa. The first hypothesis is proved, says Moss, by using the serum of group 4 to agglutinate the corpuscles of group 1, after which it would not agglutinate corpuscles of groups 2 and 3.

In discussing their conclusions as to grouping in rabbits and steers, Ottenberg and Friedman also hypothecate agglutinins and agglutinable substances. The grouping in the rabbits calls for two of each and in the steers for one of each.

My results show in all cases that agglutination seems to have occurred without any definite grouping. To account for each individual instance in the case of the swine, let us say, according to such a hypothesis, would demand a host of such receptors and agglutinins. In man, isoagglutination is distinctly a group reaction; it is constant, and it is accompanied definitely by absorption of some substances which seem to govern the reaction, so that it would seem to be due to something else than physico-chemical variations of molecular concentration. In the case of animals, rather than assume the presence of multitudinous agglutinins and receptors, it would be simpler to assume the existence of one agglutinin and one receptor only, whose actions are modified by extraneous factors, as, for instance, chemical and physical variations, perhaps infinitesimal, in the individual sera and corpuscles tested.

In conclusion, then, it appears that in man there is a distinct isoagglutinative grouping possible; that in other mammals, isoagglutination is present but according to no special order, and that in frogs, as representing amphibians, it appears to be absent. It is suggested that where facilities permit, tests be made on a wide variety of species, including monkeys and higher apes, to see whether any definite gradation exists.

## INTERAGGLUTINATION EXPERIMENTS WITH VARIOUS STRAINS OF SPOROTHRIX.\*

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Rabbits were immunized by injections of various strains of sporothrix for a period of about eight months. The animals were given at weekly intervals one slant culture which had been allowed to grow at room temperature for two weeks. The living organisms were used, at first given subcutaneously, later intraperitoneally. The animals suffered somewhat from local subcutaneous abscesses which formed at the site of injection but which never extended, and emaciation became marked in some. In the peritoneal cavity, as shown by postmortem examination, nodular masses formed on the peritoneum and about the intestines and other viscera. Involvement of the organs did not occur. The agglutinins appeared to develop slowly in the animals and tests made at the end of two months showed definite agglutination in dilutions of about 1:40 or slightly higher.

Five strains of sporothrix (A, B, C, D, and E) were used. Sporothrix A was isolated in 1909 from a typical human case which has been reported by Hyde and Davis.<sup>1</sup>

Sporothrix B was obtained by Dr. Gougerot of Paris and may be considered a typical strain of the organism as it has been observed in France and commonly called *Sporothrix Beurmanni*.

Sporothrix C was obtained by Dr. Hektoen and was isolated in 1898 from a case of typical sporotrichosis. This case was reported by Hektoen and Perkins<sup>2</sup> under the title "Refractory Subcutaneous Abscesses Caused by *Sporothrix Schenckii*."

Sporothrix D and E were obtained by Dr. K. F. Meyer of Philadelphia and were isolated from lesions on horses suffering from lymphangitis. Strain E was received too late for immunizing any rabbits.

\* Received for publication January 13, 1913.

<sup>1</sup> *Jour. Cutan. Dis.*, 1910, 28, p. 321.

<sup>2</sup> *Jour. Exper. Med.*, 1900, 5, p. 77.

These organisms are all very similar in their cultural, morphological, and pathogenic properties. French writers, especially Beurmann and Gougerot, have contended that *Sporothrix C* (*Sporothrix Schenckii*) differs sufficiently culturally and morphologically from other strains to justify a different name. This matter has been discussed at length by Gougerot<sup>1</sup> and need not be considered in detail here.

Serum was obtained from the various immune rabbits and interagglutination tests were made with the different strains and with normal rabbit serum for controls. The organisms were allowed to grow about 10 days on glucose agar and the growth was then suspended in salt solution. As a rule a pure homogeneous suspension of spores was thus obtained. Appreciable amounts of mycelial filaments were easily removed by filtering. On account of the satisfactory suspensions thus easily obtained and the large size of the elements the agglutination reactions are very readily observed and determined with definiteness. Both the macroscopic and microscopic methods were used. The latter is undoubtedly distinctly more sensitive than the former inasmuch as in practically

TABLE 1.  
AGGLUTINATION OF SPOROTHRIX A.

Immune Sera	Dilutions of Serum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Serum A.....	+++	++	++	++	+	+	o	o
Serum B.....	++	++	++	+	+	o	o	o
Serum C.....	+++	++	+	+	o	o	o	o
Serum D.....	++	++	++	+	o	o	o	o
Normal serum.....	o	o	o	o	o	o	o	o

TABLE 2.  
AGGLUTINATION OF SPOROTHRIX B.

Immune Sera	Dilutions of Serum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Serum A.....	+++	++	+++	+	o	o	o	o
Serum B.....	++	++	+++	++	+	+	o	o
Serum C.....	+++	+++	++	++	+	+	o	o
Serum D.....	+++	+++	++	++	+	+	o	o
Normal serum.....	o	o	o	o	o	o	o	o

<sup>1</sup> Kolle and Wassermann, *Handbuch der path. Microorg.*, Zweite Auflage, 1912, 5, p. 211.



TABLE 3.  
AGGLUTINATION OF SPOROTHRIX C.

Immune Sera	Dilutions of Serum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Serum A .....	+++	++	++	+	++	o	o	o
Serum B .....	+++	++	+	+	o	o	o	o
Serum C .....	+++	++	+	+	o	o	o	o
Serum D .....	+++	++	+	o	o	o	o	o
Normal serum .....	o	o	o	o	o	o	o	o

TABLE 4.  
AGGLUTINATION OF SPOROTHRIX D.

Immune Sera	Dilutions of Serum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Serum A .....	+++	+++	+++	+	+	o	o	o
Serum B .....	++++	++++	++++	+++	+	o	o	o
Serum C .....	++++	++++	++++	+++	+	+	o	o
Serum D .....	++	+++	+++	+++	++	+	+	o
Normal serum .....	+	+	o	o	o	o	o	o
	(trace)	(trace)						

TABLE 5.  
AGGLUTINATION OF SPOROTHRIX E.

Immune Sera	Dilutions of Serum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Serum A .....	+++	++	++	+	+	o	o	o
Serum B .....	+++	+++	++	++	+	o	o	o
Serum C .....	+++	+++	+++	+	o	o	o	o
Serum D .....	+++	+++	+++	++	o	o	o	o
Normal serum .....	o	o	o	o	o	o	o	o

all instances the agglutination was observed at higher dilutions. Otherwise no essential deviations were noted between the two methods. The readings given in the tables were made by the microscopic method.

An examination of the tables reveals the fact that there is a striking uniformity in the dilutions at which the different organisms are agglutinated by the various sera. The dilutions at which agglutination ceases vary with little deviation from about 1:320 to 1:640. There is in most instances a slightly higher agglutina-

tion by the sera when the homologous organisms are used but this is not always the case.

CONCLUSION.

Rabbits develop specific agglutinins for various strains of sporothrix. The several strains here tested cannot be differentiated by interagglutination.

## A STATISTICAL STUDY OF THE STREPTOCOCCI FROM MILK AND FROM THE HUMAN THROAT.\*

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### INTRODUCTION.

The English and the American work done on the classification of the streptococci by their fermentative properties on carbohydrate and related media has recently been summarized by Winslow.<sup>1</sup> He points out the deficiencies of the English method<sup>2</sup> of recording positive or negative reactions according to the color of litmus-tinted media and proposes the adoption of a uniform method of biometrical study along the lines used by certain American investigators.<sup>3</sup> The fundamental points to be observed are first, the establishing of a low and uniform initial acid reaction of the media to be tested, and second, the quantitative determination of the final acidity, using phenolphthalein as an indicator and titrating with normal sodium hydroxid. The first point mentioned is important on the supposition that acid production by the streptococci is limited only by the acidity of the medium in which they are able to grow and that, approximately, maximum acidity is reached in the time allowed for incubation. Certain tests made by us seem to verify this hypothesis but Broadhurst<sup>4</sup> obtained extraordinarily high results with milk cultures when planted in media of 1.6 per cent initial reaction. Milk was used as an isolating medium and she suggests that "this high acid production" might be considered as due to this fact. It is also a possibility that planting in a medium of high acid reaction may have enhanced the acid-producing properties of the organisms, but for this assumption we have as yet no evidence. The second point is of utmost importance in making the qualitative correlations. I believe, however, that sufficient data are now at hand to warrant an empirical end-point being established, beyond which the reaction shall be recorded as positive fermentation. This point should be established not by any empirical reaction of an indicator to acid but by a study of the results according to biometric principles. Previous work by Winslow seemed to indicate that the streptococci tend to distribute themselves about two modes, the valley or gap occurring at about 0.8-1.0 per cent acid. Reference to the accompanying charts and tables further substantiates the

\* Received for publication December 20, 1912.

<sup>1</sup> *Jour. Infect. Dis.*, 1912, 10, p. 285.

<sup>2</sup> Gordon, M. H., *Thirty-third Ann. Rept. of Local Govt. Bd.*, 1905; *Rept. on Investigation of Ventilation of Debating Chamber of House of Commons*, 1906; and *Fourteenth Ann. Rept. of Local Govt. Bd.*, 1910-11; Houston, A. C., *Rept. of London County Council*, 1905; Andrews, P. W., and Horder, T. J., *Lancet*, London, 1906, 171, p. 708.

<sup>3</sup> Winslow and Rogers, *Relationships of the Coccaceae*, 1910; Winslow and Palmer, *Jour. Infect. Dis.*, 1910, 8, p. 1; Broadhurst, Jean, *Jour. Infect. Dis.*, 1912, 10, p. 272; Stowell and Hilliard, *Amer. Jour. Dis. of Children*, 1912, 3, p. 287; Morse, M. E., *Jour. Infect. Dis.*, 1912, 11, p. 253; Howe, E. C., *Science*, 1912, 35, p. 225; Rogers and Davis, *ibid.*, p. 230; *Bur. of Animal Ind. Bull.* 154, 1912; Browne, W. W., *Science*, 1912, 35, p. 236.

<sup>4</sup> *Loc. cit.*

previous observations. Winslow has suggested that the division point between acid production and non-acid production "may be placed without serious error at 1.2 per cent normal" acid and in the following discussion we shall use this as the dividing line.

#### MATERIAL.

We have studied 240 strains of pure cultures of streptococci isolated from 75 separate sources. All cultures used were isolated by one of us so that we know in every case the complete history, age, source, etc., of the organism. Organisms have been isolated from two sources: from cow's milk (55 strains) and from the human throat (185 strains). In accepting more than one strain from a source, we always attempted to take only those showing different cultural or morphological features in the preliminary work of isolation. At times only one culture was obtained from a given source, and at other times as many as five were accepted. We practically never found it impossible to isolate streptococci either from throat swabs or from city milk, suggesting the ubiquitousness of the streptococcus. Fresh milk collected at the dairy or soon after milking was often free from streptococci.

The work has been done in three distinct stages, and hence certain tests have been added in the latter part of the work that were not used in the beginning. For this reason our percentage comparisons that are given in the further discussion are a bit misleading. The following table will assist in clarifying this discrepancy.

TABLE I.  
SHOWING THE NUMBER OF STRAINS USED IN EACH TEST AT THE TWO TEMPERATURES.

The Test	Number Strains Tested		Number Strains not Tested	
	37° C.	20° C.	37° C.	20° C.
Dextrose.....	240	110	0	130
Lactose.....	240	110	0	130
Saccharose.....	240	110	0	130
Salicin.....	174	44	66	196
Raffinose.....	240	110	0	130
Inulin.....	174	44	66	196
Mannite.....	189	110	51	130
Morphology.....	240	...	..	...
Gram Stain.....	240	...	..	...
Violet positive test.....	87	...	..	...
Hemolysis.....	95	...	..	...

It is apparent from this table that the salicin and inulin fermentation tests were added after the work was started, and the

violet positive test (first reported by Churchman<sup>1</sup> in July 1912) and hemolysis tests were done in less than half the total number of strains.

#### METHODS.

**Isolation.**—The throat specimens were taken on sterile absorbent cotton swabs from the back of the throat. The swabs were then washed off in sterile water tubes and a few drops were immediately plated on nutrient agar made according to standard methods. The milk samples were diluted in sterile water and plated with agar in the usual way. The plates were examined at the end of 36 hours and small, round, white surface colonies, or small, lenticular, subsurface colonies, characteristic of streptococcus growth on this medium, were fished and inoculated into standard nutrient broth. These tubes were examined at the end of 24 hours for growth typical of streptococcus and likely cultures were then examined microscopically in smears stained by Gram's method. Morphology, and purity of culture were thus determined. Cocci occurring three or more in a chain were accepted; diplococci were eliminated. Cocci in pairs within a long chain we admitted. Cultures showing chains containing more than eight elements were classed by us as "long chained." The oval, chained form—*S. lacticus* of Kruse, really of the *B. lactis acidi* group—was never accepted by us. The accepted strains were inoculated from broth into North's medium<sup>2</sup> and were transferred to fresh tubes of North on every third day, until put through the routine tests. Twenty-four-hour-old cultures grown on this gelatin agar medium were always used for the tests and usually they were transferred only once while on this medium before being run through.

**Inoculation.**—The inoculations into the carbohydrate media to be tested were made with a platinum loop of approximately 4 mm. diameter. Luxuriant growth usually appeared in the water of condensation of the North tube.<sup>3</sup> This was thoroughly mixed, together with whatever surface growth there might be, and then the biconcave drop was lifted with the loop and thoroughly washed off in the carbohydrate broth tube. Duplicate tubes were always inoculated for all tests.

**Preparation of media.**—Nutrient broth was made in the usual way, using 3 gms. of Liebig's beef extract to the liter. This was made sugar free by inoculating with a pure culture of *B. coli* and incubating. We believe from the experience we have had that this precaution is wholly superfluous when broth is made with the extract, as we obtained only from 0.0 to 0.2 per cent rise in acid after inoculation with coli. To the sugar-free broth, 1 per cent by weight of the test carbohydrate substance was added just before tubing. All test media were sterilized by intermittent sterilization in streaming steam on three successive days. The final reaction of the medium varied from 0.0–0.3 per cent acid.

**Incubation.**—The duplicate inoculated tubes were incubated at 37° or 20° C., as the case might be, for three days. This time has been found by our own experience and that of others to give approximately maximum acidity with the streptococci.

<sup>1</sup> *Jour. Exper. Med.*, 1912, 6, p. 221.

<sup>2</sup> North medium is made with 1 per cent agar and 3 per cent gelatin. It has been suggested by C. E. North for use with pneumococci and streptococci especially. The unusually luxuriant growth of streptococcus and the abundant water of condensation at the base of the slant, from which we could transfer uniform amounts with a loop, made this media especially valuable for our purpose.

<sup>3</sup> Heavier growth may be obtained by adding from a trace to 1 per cent glucose to the North medium.

With the diphtheria group, on the other hand, a longer time has been found necessary for incubation. Two blank tubes were always incubated at the same time for controls.

*Titration.*—Five c.c. of the inoculated broth were drawn off with a graduated pipette and this amount was drained into a white porcelain evaporating dish, diluted with 45 c.c. of distilled water and titrated cold, against N/20 sodium hydroxid, using phenolphthalein as an indicator. The first indication of permanent pink coloration was taken as the end-point. The pipette was thoroughly washed with distilled water before the next culture was tested. We found it more convenient, toward the end of our work, to run our broth into the test tubes from burettes in carefully measured 5.1 c.c. amounts. When ready for acid determination, the tubes are simply drained into the evaporating dishes. We believe that an accurate amount of culture media in which the organisms are permitted to grow may also give slightly more uniform results.

#### RESULTS.

*Fermentation.*—Armstrong<sup>1</sup> says that "The process of fermentation of a sugar is regarded as a series of consecutive reactions each involving simplification of the sugar molecule till it breaks down into carbon dioxid and ethyl alcohol, compounds containing only one and two carbon atoms." The streptococci never carry a fermentation through to the simple elements  $\text{CO}_2$  and  $\text{C}_2\text{H}_5\text{OH}$ , so that we must make our measurements of the amount of fermentation activity that they exert by determining the amount of acid they split off from the molecule. A chemical molecule must be conceived as a ring of links with here and there a "side chain." As with any combination of links, the strength of the series is represented by the strength of the weakest link, or it is possible that one or more of the "side chains" may be more easily disunited than the others by specific bacterial activity. When the breakdown of the molecule begins, it is always at these weakest points. It is this varying capacity of different bacterial strains to separate the uniting bonds existing between atoms or groups of atoms and thus to avail themselves of the energy liberated in the process, which we try to measure by acid titration.

*Metabolic gradient.*—Previous work done by two of us on the genus streptococcus and by Howe<sup>2</sup> on the colon group seemed to suggest a very interesting relationship existing between the geometric configuration or the size of the molecule of carbohydrate and the availability of the substance for the organism. Winslow emphasized this "order of relative availability" and pointed out

<sup>1</sup> *The Simple Carbohydrates and the Glucosides*, p. 53.

<sup>2</sup> *Op. cit.*

that if "any member of the series is fermented, the chances are that those ahead of it will be fermented also." Howe has aptly

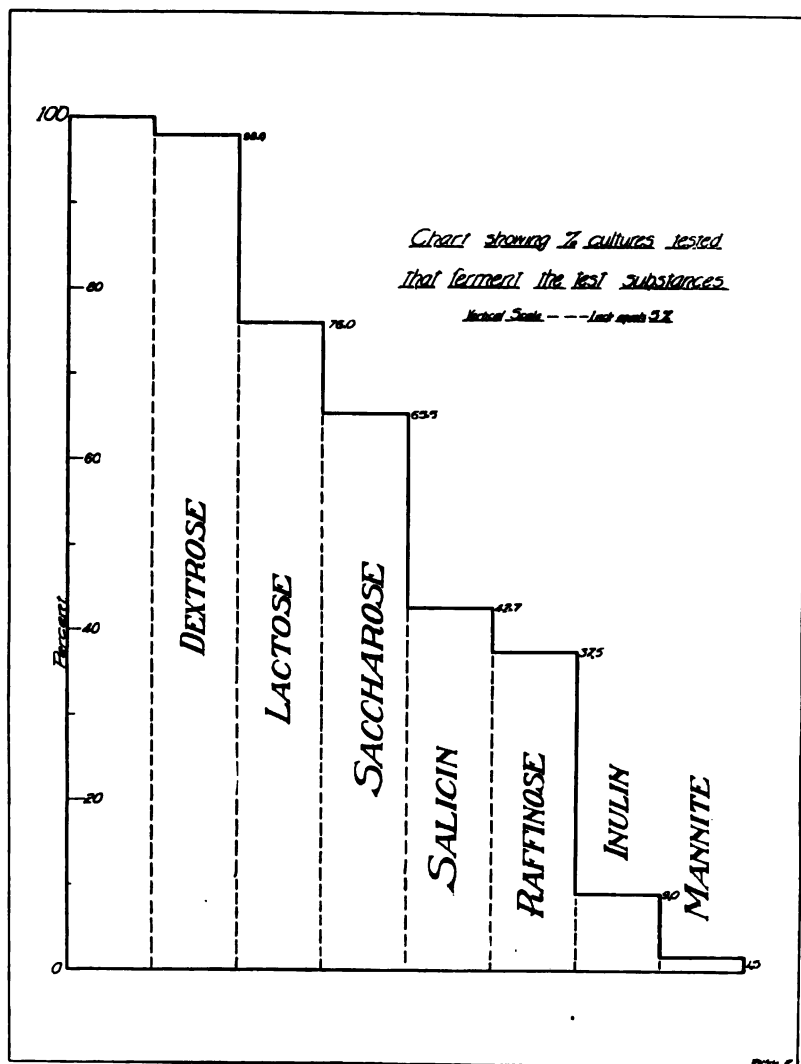


FIG. 1. Showing percentage of cultures that ferment the test substances.

suggested the phrase "metabolic gradient" to represent this phenomenon.

Fig. 1 shows graphically this gradient as found with the organisms and substances used in this study. Winslow in correlating the results of several workers finds the following order to exist:

Substance	Percentage Positive Results
Dextrose.....	82
Lactose <sup>1</sup> .....	65
Salicin.....	75
Saccharose.....	55
Inulin.....	32
Mannite.....	18
Raffinose.....	12

Our order, it will be observed, is quite different, especially as regards the position of raffinose and salicin in the gradient. These differences are accounted for, first, because of the very small number of saccharose-salicin results used, and second, owing to the large number of faecal cultures included, which ferment mannite and do not ferment raffinose, thus inverting the order of these two substances. Winslow's compilation, being made from the data of a number of workers and from strains isolated from different sources, claims only to be tentative. Our figures have been obtained by a uniform method and the organisms have come from only two sources and we believe that it is wholly logical, as we shall attempt to show.

Table 2 shows the individual correlation of our results arranged as in the Winslow paper referred to. The lower left-hand figure represents what may be termed the aberrant group, i.e., those not reacting in the order with the majority. It will be noted that only in two instances is this group large: in the lactose, saccharose, and raffinose squares and in the salicin-raffinose square. An organism similar to the former group that fails to ferment lactose and yet splits up the higher substances saccharose and raffinose has been described as *S. equinus* by several workers. The group skipping salicin and using raffinose has also been described by Andrews and Horder (see Table 4). Reference to Fig. 1 shows that salicin and raffinose are fermented by about the same number of organisms and the table under discussion shows that if the order were reversed only 20 strains would fall

<sup>1</sup> Put before salicin because the small number tested on salicin did not justify putting it second.



under the bane of aberrant. The order with these two substances is only tentative.

TABLE 2.  
CORRELATIONS OF DIFFERENT ORGANIC MEDIA AS REGARDS THEIR LIABILITY TO FERMENTATION.

	Lactose		Saccharose		Salicin		Raffinose		Inulin		Mannite	
	+	-	+	-	+	-	+	-	+	-	+	-
<i>Dextrose</i> —												
+.....	189	53	166	71	71	99	87	150	15	158	2	181
-.....	0	5	0	5	0	3	0	5	0	3	0	5
<i>Lactose</i> —												
+.....	...	...	146	38	70	74	75	100	13	138	2	128
-.....	...	...	22	36	11	28	22	46	0	39	0	59
<i>Saccharose</i> —												
+.....	...	...	...	...	65	59	84	80	11	113	2	138
-.....	...	...	...	...	6	43	5	73	2	47	0	49
<i>Salicin</i> —												
+.....	...	...	...	...	...	...	53	20	9	62	2	24
-.....	...	...	...	...	...	...	29	71	4	98	0	92
<i>Raffinose</i> —												
+.....	...	...	...	...	...	...	...	...	11	71	1	51
-.....	...	...	...	...	...	...	...	...	2	89	1	136
<i>Inulin</i> —												
+.....	...	...	...	...	...	...	...	...	...	...	1	9
-.....	...	...	...	...	...	...	...	...	...	...	1	111

The accompanying dot chart brings out still more strikingly this food relationship of the streptococci. The position of each strain in its relation to each of the seven substances is represented by a dot (or circle) in the vertical columns. The center of population, as it were, for the substance-acid relationship has been determined and a line has been plotted through these centers (the heavy line). Thus the center of distribution of all the strains when grown in glucose lies at about 3.15 per cent and gradually approaches the zero point with the least available substance, mannite. Note the absence of strains falling between 0.4 and 1.2 per cent, this representing the valley that divides the positive (fermenting) and the negative (non-fermenting) organisms referred to above.

"The order of availability, so far as the streptococci are concerned, corresponds closely to what might be expected from the chemical composition of the substances."<sup>1</sup> We will consider briefly the chemical properties of the substances used to see whether our "metabolic gradient" is logical:

<sup>1</sup> Winslow, *loc. cit.*

**Dextrose.**—A monosaccharid ( $C_6H_{12}O_6$ ) of the formula  $CHO.(HCOH)_4.CH_2OH$ . Glucose shows a great tendency to become further oxidized, evidenced by its activity as a reducing agent. It contains the aldehyd radical. On oxidation it gives rise to gluconic, glucuronic, and saccharic acid.

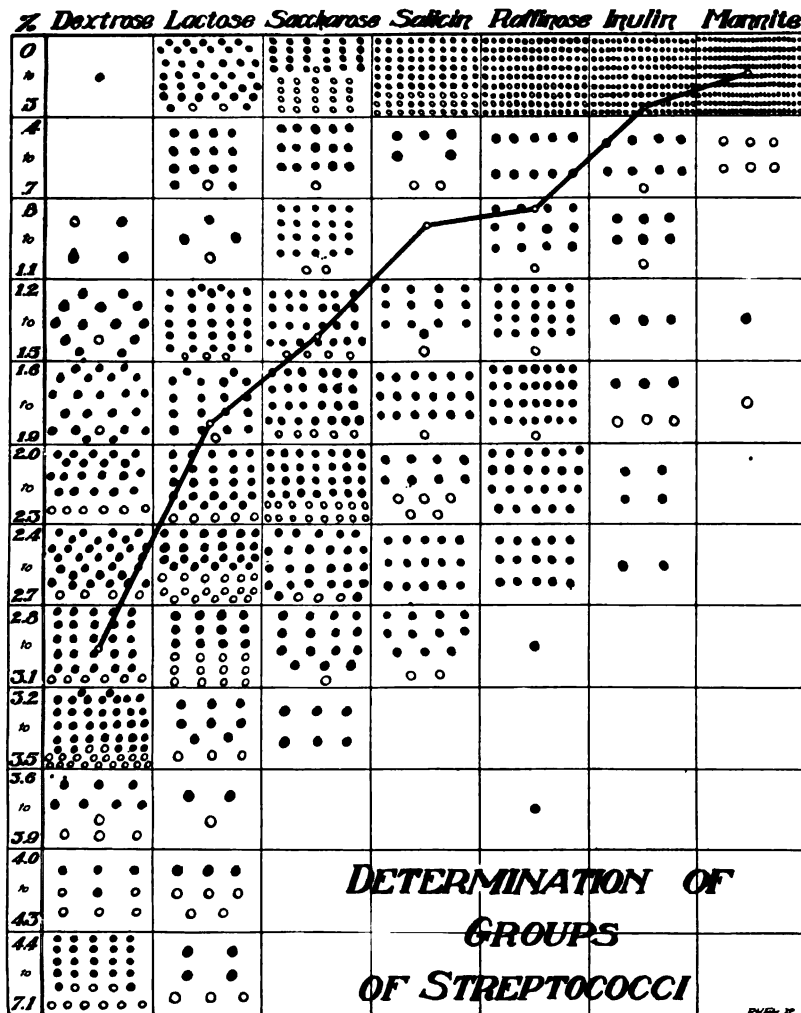


FIG. 2. Showing the distribution of the individual strains and the groups into which the milk and throat streptococci fall, according to their capacity to ferment the test substances. The line is drawn through the mathematical center of distribution.

**Lactose.**—A disaccharid ( $C_{12}H_{22}O_{11}$ ). The stereo-chemical configuration is not definitely decided upon but it is probably made up of a galactose and glucose residue united by a primary alcohol group. The aldehyd group is potentially functional. Upon fermentation it yields, especially, lactic and butyric acid.

**Saccharose.**—Sucrose or cane sugar is a disaccharid ( $C_{12}H_{22}O_{11}$ ). Fischer's formula pictures it as made up of a glucose and a fructose residue so united as to destroy both the aldehyd and ketone groups, thus giving it only neutral properties. It is readily hydrolized by dilute mineral acids or by the action of the ferment invertase by inversion, yielding a mixture of equal parts of glucose and fructose, invert sugar.

**Salicin.**—A glucosid of the formula  $(C_6H_{11}O_5 \cdot O) \cdot C_6H_4 \cdot OH_2OH$ . The glucosids include a large number of substances found in plants having the common property of furnishing glucose and one or more organic residues when hydrolized. Salicin is ultimately oxidized to salicylic acid and the acids formed by the oxidation of dextrose.

**Raffinose.**—One of the commonest trisaccharids ( $C_{18}H_{32}O_{16}$ ). Armstrong gives the following constitutional formula:  $C_6H_{11}O_5 \cdot O \cdot C_6H_{10}O_4 \cdot O \cdot C_6H_{11}O_5$ . It has no reducing properties and behaves chemically very much as sucrose. It is hydrolized by mineral acids into melibiose and fructose and upon further oxidation may form a variety of substances.

**Inulin.**—Inulin is a soluble starch compound ( $C_6H_{10}O_5$ )<sub>n</sub>. Heated with mineral acids it yields fructose.

**Mannite.**—A hexahydric alcohol  $C_6H_8(OH)_6$  or  $CH_2(OH) \cdot (CH \cdot OH)_4 \cdot CH_2OH$ . It has the usual properties of an alcohol. It is closely related to the sugars yielding levulose on oxidation.

It has been pointed out that with the streptococci the size or bulk of the molecule is the controlling factor and that with the coli the stereo-chemical configuration determines whether the substance shall be available or not.<sup>1</sup> We believe that with the streptococci both factors are important as a study of the summary discussion above and the percentage of organisms that ferment each will tend to show. To determine this metabolic relation more exactly we should work with substances more closely related, e.g., with the disaccharids only as maltose, sucrose, lactose, cellobiose, melibiose, etc.

We may now correlate our fermentation results to determine, if possible, a biometrical classification of the streptococci on this basis. The following table shows that 209 strains (90 per cent) of all the cultures may be classified in nine groups by the use of the seven test substances when incubated at 37° C.

We shall leave the study of the comparative reactions of the throat and milk organisms for a subsequent division of the paper and will discuss only the group reaction. There is a small group—five out of 240—that are wholly incapable of fermenting the most simple substance, dextrose. Still, we must consider it as a specific group as reinoculations proved the organisms capable of growth

<sup>1</sup> Howe, *op. cit.*; Jackson, *op. cit.*

and limited growth appeared in the sugar tubes, but none, or less than 1.2 per cent acid, was produced. The second group is as yet undescribed so far as we can learn. Many workers have neglected to use glucose as it is so uniformly fermented by the streptococci, and therefore the fact that there is quite an important group with the limited capacity to attack this substance only has been

TABLE 3.

CLASSIFICATION OF THE MILK AND THROAT STREPTOCOCCI ACCORDING TO POSITIVE OR NEGATIVE FERMENTATION OF THE TEST SUBSTANCES AT 37° C.

SOURCE OF CULTURE	NUMBER OF CULTURES	PERCENTAGE		GROUP	DEXTROSE	LACTOSE	SACCHAROSE	SALICIN	RAFFINOSE	INULIN	MANNITE
		Part	Total								
Throat...	5	2.7	2.	I	—	—	—	—	—	—	—
Milk.....	0	0.									
Throat...	27	14.5	12.	II	+	—	—	—	—	—	—
Milk.....	4	7.4									
Throat...	17	9.2	10.6	III	+	+	—	—	—	—	—
Milk.....	13	24.									
Throat...	31	16.7	22.5	IV	+	+	+	—	—	—	—
Milk.....	23	42.5									
Throat...	10	7.5	5.8	V	+	+	+	+	—	—	—
Milk.....	4	10.3									
Throat...	32	23.8	73.3	VI	+	+	+	+	+	—	—
Milk.....	0	0.									
Throat...	9	6.7	3.7	VII	+	+	+	+	+	+	—
Milk.....	0	0.									
Throat...	10	7.5	4.1	VIII	+	—	+	+	+	—	—
Milk.....	0	0.									
Throat...	22	12.	10.	IX	+	+	+	—	+	—	—
Milk.....	2	3.7									

overlooked. Most investigators have used lactose litmus agar for an isolation medium, which has resulted in a selection of forms that at least ferment lactose, and so they have entirely overlooked organisms fermenting a lower substance or with no fermentative properties. We believe that this substance should always be included in the series, as 17 per cent at least will otherwise evade classification or be misplaced.

Continuing the examination of the table, we find that more complex substances are successively used by a series of groups through inulin. The large group is the dextrose-lactose-saccharose fermenter. Forty-five per cent of the total number may be classified in groups II, III, and IV. Group V includes a small number—14—because of the similar availability of salicin and raffinose already discussed. Group VI is the second largest, fermenting without skipping a substance, through raffinose. We ought to

note in passing that we find not a single milk strain included here. The next group, embracing nine strains of throat streptococci, is the most adaptable of all, and ferments without fail every test substance but mannite. We believe that enough work has been done, on throat organisms at least, to warrant the exclusion of mannite as a test substance in future work. It is invariably a negative substance, the only two instances in which it was positive occurring in aberrant groups.

The following table gives our results in comparison with other classifications of the genus streptococcus by the capacity they show to ferment the Gordon test substances:

TABLE 4.  
SHOWING COMPARATIVE CLASSIFICATIONS OF THE STREPTOCOCCI BY THE BIOMETRIC-FERMENTATION METHOD ACCORDING TO THE NUMBER OF SUBSTANCES FERMENTED.

Investigator	Organism	Lactose	Saccharose	Salicin	Raf- finose	Inulin	Man- nite
Gordon	.....	..	..	..	..	..	..
Andrews and Horder	.....	..	..	..	..	..	..
Broadhurst	A. ....	—	—	—	—	—	—
Stowell, Hilliard, and Schlesinger	II. ....	—	—	—	—	—	—
Gordon	.....	..	..	..	..	..	..
Andrews and Horder	.....	..	..	..	..	..	..
Broadhurst	B. ....	—	—	+	—	—	—
Stowell, Hilliard, and Schlesinger	III. ....	+	—	—	—	—	—
Gordon	.....	+	+	—	—	—	—
Andrews and Horder	1b, 3, and 4s. <i>S. equinus</i> ..	—	+	+	—	—	—
Andrews and Horder	<i>S. anginos us</i> ..	+	+	—	—	—	—
Broadhurst	C. ....	+	—	+	—	—	—
Stowell, Hilliard, and Schlesinger	IV. ....	+	+	—	—	—	—
Gordon	.....	..	..	..	..	..	..
Andrews and Horder	<i>S. pyogenes</i> , <i>S. mitis</i> ....	+	+	+	—	—	—
Broadhurst	D. ....	+	+	+	—	—	—
Stowell, Hilliard, and Schlesinger	V. ....	+	+	+	—	—	—
Gordon	.....	+	+	—	+	—	—
Andrews and Horder	1a <i>S. salivarius</i> ..	+	+	—	+	—	—
Broadhurst	.....	+	+	—	+	—	—
Stowell, Hilliard, and Schlesinger	IX. ....	+	+	—	+	—	—
Stowell, Hilliard, and Schlesinger	VIII. ....	—	+	+	+	—	—
Gordon	.....	+	+	+	+	—	—
Andrews and Horder	4b <i>S. faecalis</i> ..	+	+	+	—	—	+
Broadhurst	E. ....	+	+	+	—	+	—
Stowell, Hilliard, and Schlesinger	VI. ....	+	+	+	+	—	—
Gordon	.....	..	..	..	..	..	..
Andrews and Horder	.....	+	+	+	+	+	+
Broadhurst	F. ....	+	+	+	+	+	+
Stowell, Hilliard, and Schlesinger	VII. ....	+	+	+	+	+	—

The Gordon results (Table 4) show his groupings for organisms isolated from normal throats. The Andrews and Horder are the general

groupings shown by a large number of strains from various sources. The Broadhurst figures show the largest groupings appearing in work on 100 milk strains of streptococci.

It will be noted that we have used only six test substances in the above table, omitting dextrose and hence eliminating our first group. This comparison does not show any great uniformity in results and is not at first glance encouraging to the theory that there is a true and stable metabolic gradient. Could great uniformity be expected when we consider the difference in the English and the American methods of obtaining results? The Gordon, and the Andrews and Horder results indicate only whether a litmus-tinted medium, originally faintly alkaline (to litmus), has been reddened or not. It has been found that when results obtained by the quantitative method described above are plotted "they usually show two distinct maxima, one in the neighborhood of the point of no acid formation, the other at a somewhat high acidity."<sup>1</sup> The litmus method would seem to be less accurate than the titration method, and as the end-point of litmus—0.8—is somewhat lower than 1.2 per cent (our standard), we might expect that the English results would be somewhat higher, and this, Winslow has found to be the case. Also, a rough correlation of results might be expected, as the two end-points are fairly close together, and this again seems to be the case.

We must examine this table (Table 4), especially, bearing in mind the source of the organisms used. Gordon's results with throat streptococci agree with ours in two instances: Groups IV and IX. Table 3 shows these groups to contain 16.5 per cent and 12 per cent respectively of our throat strains. The Andrews and Horder results agree with ours in three instances. Their classification is based on the study of about 1,200 strains from various sources. *S. anginosus* corresponds to our group IV, fermenting lactose and saccharose. Our group V checks with two species named by Andrews and Horder: *S. mitis* and *S. pyogenes*. These strains are described as identical in their fermentative properties but vary in their morphology. Broadhurst also checks on this group. The very interesting form, *S. salivarius*, which ferments

<sup>1</sup> Winslow, *op. cit.*

raffinose and not salicin, has been described by both the English workers. We find only two milk strains classified here and Broadhurst finds none, indicating that this is a specific throat form. The *S. equinus*, which is aberrant to lactose, is described as fermenting only saccharose and salicin. We are inclined to believe that the 10 organisms in group VIII are identical with *S. equinus*, though of course it may be a new form when the origin of these strains is considered. Broadhurst in the work on milk streptococci finds the group II fermenting none of the six sugars used—relation to dextrose unknown—and also V, mentioned above.

Our analysis of the table shows then only three strains that are comparably unaccounted for. Group III ferments lactose only. It is a large group containing representatives from both sources and seems to be definitely limited in its fermentative power. Groups VI and VII are specific throat forms with unusual ability in splitting up the higher substances. They are large enough to be representative and are forms that might logically be expected to be found according to the gradient theory. Our group I is not comparable with other results for reasons previously indicated but we believe it is a group that should not be neglected in the future by the selective methods of isolation previously used by many workers. Groups IV, V, and IX, including about half of the throat organisms classified, represent the only three species described by Andrews and Horder, which, it seems to us, is a very powerful correspondence. We would not expect to find *S. faecalis*.

D. H. Bergey<sup>1</sup> has compared 92 cultures from a variety of sources, using the English method. He finds all of the Andrews and Horder groups except the *faecalis*. There seems to be little correlation between the source from which the organism came and its identity. For example, out of 16 cultures showing the fermenting properties of *S. equinus*, half came from horse manure and eight from the human throat. We are also inclined to question the capacity of *S. mitis* to ferment inulin and its failure to ferment saccharose; and of *equinus* to ferment salicin, according to the original intentions of Andrews and Horder. *Salivarius* and *anginosus* are grouped together by Bergey but we believe that a general negative

<sup>1</sup> *Jour. Med. Research*, 1912, 27, p. 67.

raffinose result with the latter organism is now established. On the whole, however, the work substantiates the food relationships of the streptococci when studied by the biometric method.

When the broths are incubated at the room temperature we find a very marked change in the gross grouping of the streptococci in their relation to the test substances. We find that 89 out of the 110 strains studied, or about 81 per cent, may be grouped around four reactions in sugars. The following table shows this relationship. Two throat strains only show the capacity to ferment any substance other than a single sugar when incubated at 20° C.

TABLE 5.

CLASSIFICATION OF MILK AND THROAT STREPTOCOCCI ACCORDING TO POSITIVE OR NEGATIVE FERMENTATION OF THE TEST SUBSTANCES AT 20° C.

SOURCE OF CULTURE	NUMBER OF CULTURES	PERCENTAGE		GROUP	DEXTROSE	LACTOSE	SACCHAROSE	SALICIN	RAFFINOSE	INULIN	MANNITE
		Part	Total								
Throat...	38	64.5	35.4	Ia	-	-	-	-	-	-	-
Milk.....	1	2.0									
Throat.....	14	23.9	17.2	IIa	+	-	-	-	-	-	-
Milk.....	5	10.									
Throat.....	1	1.7	15.4	IIIa	+	+	-	-	-	-	-
Milk.....	16	32.									
Throat.....	1	1.7	12.7	IVa	+	+	+	-	-	-	-
Milk.....	13	26.									

The importance of this table does not lie so much in the groups shown as in the striking differentiation between the organisms from the two sources, throat and milk. The capacity to ferment any sugar other than dextrose seems to be wholly denied streptococci from the throat while 56 per cent of all the strains isolated from milk flourish on lactose media.

#### COMPARISON OF THE MILK AND THE THROAT STREPTOCOCCI.

This leads us to a brief consideration of differential fermentative features of the streptococci from the two sources, cow's milk and the human throat. The streptococci isolated from milk were taken in part from fresh milk collected at the dairy or from the milk wagon on a short country route, and from samples of dirty city milk.

Reference to Tables 3 and 5 shows that there are certain groups that are exclusive of either milk or throat organisms. Thus at



37° C. groups VI, VII, and VIII—using substances beyond salicin—contain no milk streptococci at all. So far as our work is concerned we may call these, distinctly throat groups. At 37° C. group IV is most specific for milk strains, containing 42 per cent of the total. Table 3 shows that groups III and IV are distinctly milk groups.

In a previous paper of ours we have noted the fact that "throat streptococci do not readily ferment at 20° any of the sugars used, while the milk organisms attack the same sugars and to the same extent at this temperature as at 37° C." Our further studies have shown this preliminary conclusion still to be the most striking differential feature. Reference to the accompanying curves shows very positively this difference in temperature-fermentation relation.

Reference to Fig. 2 or to the classification tables will show this same thing in a different way. It is very obvious that the throat streptococci are less fastidious as to the substance they ferment but are very susceptible to temperature relations, while the milk strains very seldom give a positive acid reaction when grown in the presence of a substance higher in the series than saccharose but ferment through this substance equally well at either 20° or 37° C.

The reactions are so constant and so different between the strains from the two sources that we feel justified in suggesting certain empirical standards for identifying the original source of unknown strains. We believe that the following features are sufficient to separate milk from the throat streptococci: (1) they yield over 2.5 per cent acid in lactose and saccharose at 37° C.; (2) they seldom ferment a substance higher than saccharose in the metabolic series; (3) they readily grow in dextrose, lactose, and saccharose at 20° C. Throat streptococci, on the other hand, (1) seldom yield over 2.5 per cent acid in any substance at any temperature; (2) over 40 per cent yield over 1.2 per cent acid in either salicin or raffinose or in both at 37° C.; (3) at 20° C. they almost never attack any of the test substances.

Reduced to still lower terms we would say that six different tubes of media would place an unknown streptococcus if it came from one of the sources studied: dextrose, lactose, raffinose, and

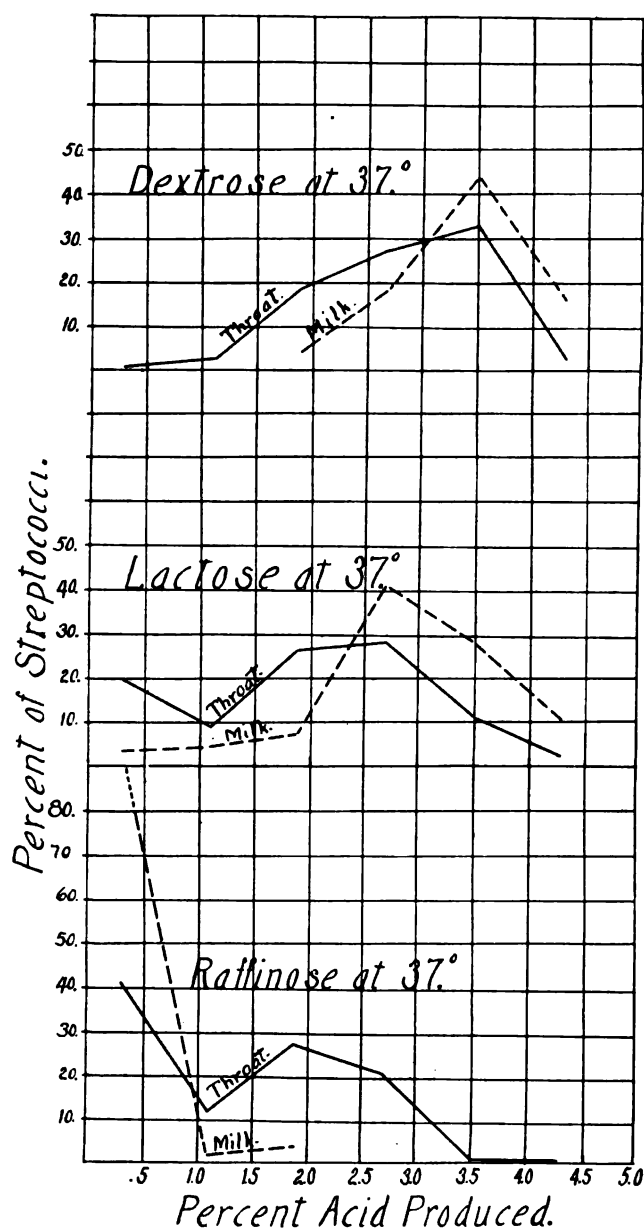


FIG. 3.—Curves of dextrose, lactose, and raffinose, showing the percentage of cultures from each source in relation to the percentage of acid formed when grown at 37° C.

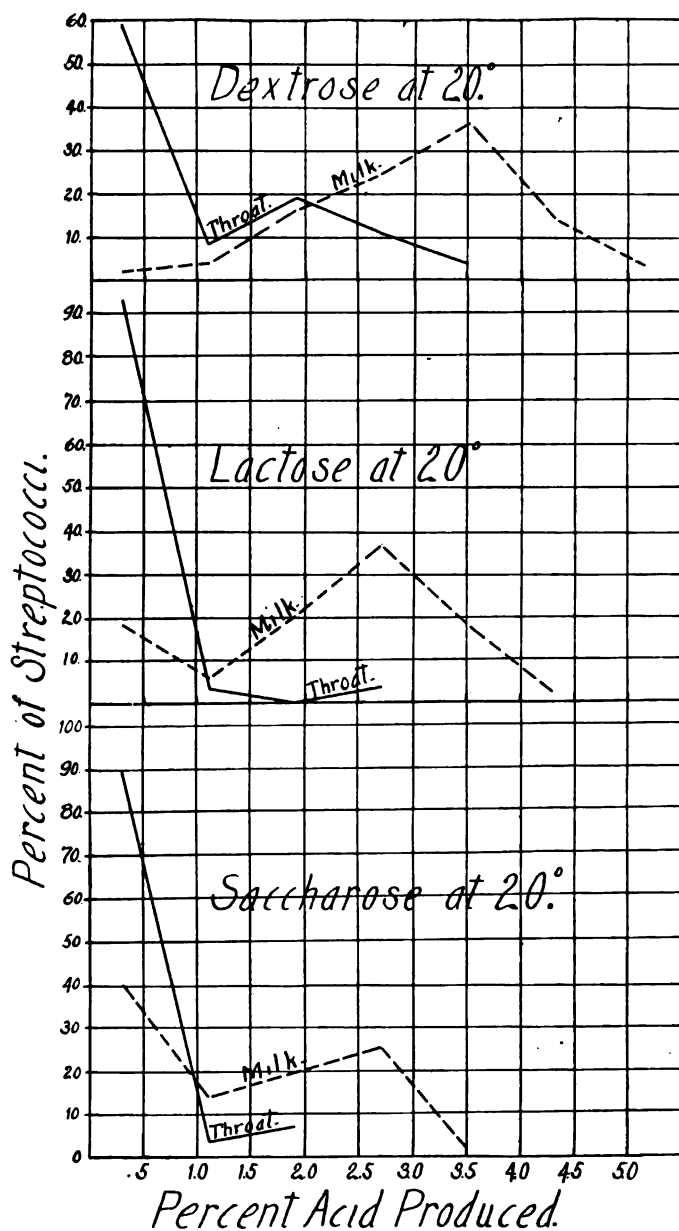


FIG. 4.—Curves of dextrose, lactose, and saccharose, showing same relation as Fig. 3 when grown at 20° C.

salicin at 37° C., lactose and saccharose at 20° C. With a milk strain we expect above 2.5 per cent acid in dextrose and lactose at 37° C.; negative fermentation in the other two media at that temperature; fermentation in the two substances incubated at 20° C. A strain from the throat would be expected to give lower acid production in all tests, should ferment salicin or raffinose, and should give little or no acid when incubated at 20° C. in the two substances suggested. Four of the fixed tests would be considered diagnostic.

#### FEATURES OTHER THAN FERMENTATION STUDIED.

*Morphology.*—We examined most of our cultures twice microscopically to obtain, if possible, some correlation between the length of chain, size of the coccus, etc., and other features. We were unable to group these with any other characteristics. The only observation worthy of note is that only about 25 per cent of all the throat cultures were long chained, i.e., 10 cells or more, while about 75 per cent of the milk organisms were grouped by us as "long."

*Gram stain.*—We carefully stained 24-hour cultures on North media by the Gram method in the hope that this reaction might prove of diagnostic value. We found that we could make no correlation with this stain reaction and any other characteristic. The large majority of the cultures retained the stain, a few stained irregularly, and a few were wholly decolorized.

*Violet positive test.*—Churchman recently reported a study of the "bacteriostatic" properties of gentian violet when added to media in minute doses. He found that there was a very consistent parallelism between this inhibiting property of the stain in media and the Gram stain. Following his technic of divided plate, we tested 87 of our cultures. All but five strains refused to grow on the violet side of the plate while all grew well on the plain agar control side. Included in those that were "violet positive," i.e., those that did not grow, were several gram-negative strains. The parallelism with the coccaceae is apparently not absolute, as is pointed out by Churchman. The test is of no differential value in this study.

*Hemolysis.*—The hemolytic property of 92 strains was studied in the following manner: A few drops of fresh, sterile rabbit's blood were added to cooled melted agar tubes and petri plates were then poured. After incubation of the plates for 24 hours, freshly isolated cultures were streaked on the plate with a small, light, platinum loop. The plates were examined after 24 hours at 37° C. and if there was any hemolysis the clear area was measured and recorded in millimeters. The plates were usually kept for a longer period to see if any further changes took place, but our records were kept on the 24-hour reading.

Of the 92 strains so tested, 17 gave hemolysis. The breadth decolorized varied in extent from 0.5–2.5 mm. Five of the 17 giving a positive hemolytic test were from "normal" milk, five—the most vigorous—from milk of dairies where udder trouble was known to exist, and seven were from normal throats. In three instances the "greenish" color described by Ruediger<sup>1</sup> as characteristic of the *S. lacticus* was noted. The capacity to hemolyze rabbit's blood during the short incubation period allowed is not correlated with any other of the characteristics studied, so we have neglected it in our groupings. As a single cultural feature to be studied in connection with pathogenicity, it is invaluable, as has been shown by other workers.<sup>2</sup>

*Character and amount of growth.*—The character and the luxuriance of growth on North medium and in broth have been studied by us but we have been able to make no correlations worthy of discussion. The length of chain and consistency and cloudiness of the broth are correlated. In the test broths the amount of growth varied almost directly with the degree of acidification. We may note here that zero acid indicates no growth almost invariably. In other words, the substances which are not fermented act as inhibiting or "bacteriostatic" agents and are non-available for use. It is possible that in certain instances it acts even as a germicidal agent, though we did not make any extended research into the question. Reinoculations after the three days'

<sup>1</sup> *Op. cit.*

<sup>2</sup> Jupille, F., *Ann. de L'Inst. Past.*, 1911, 25, p. 918; Ruediger, G. F., *Amer. Jour. Pub. Health*, 1912, 2, p. 107; Davis, D. J., *Jour. Amer. Med. Assoc.*, 1912, 58, p. 1851.

incubation frequently found the tubes sterile but doubtless the bacteria, being unable to reproduce, had died from old age in this time. We are positive that no growth did not mean failure to inoculate the tubes, as our duplicates always checked with remarkable constancy and in most instances where they did not check, i.e., growth in one tube and none in the other, we confirmed the test by reinoculating in duplicate a second time.

*Stability of the fermentation tests.*—Frequently, where we were doubtful of a result, we inoculated fresh tubes a week or even longer after the first series had been run. Such tests usually confirmed our first finding. At one time we ran 72 strains through raffinose several weeks apart and the reactions checked closely. The first 50 cultures we worked with were not of uniform age when passed through the various test substances and we ran them all through a second time several weeks later. (A few of the cultures had died in the interim.) With hardly an exception they confirmed the first records. We believe, therefore, that the fermentative properties of the streptococci, exclusive of pathogens, are reasonably stable when kept under cultivation and hence may be depended upon to remain constant in their normal environment. Rogers and Davis<sup>1</sup> have recently noted the same fact. This feature, of course, lends value to our general thesis that the fermentative tests may be depended upon as of diagnostic value.

#### SUMMARY AND CONCLUSIONS.

Two hundred and forty pure strains of streptococci isolated from milk and from the normal human throat have been compared as to their morphology, gram stain reaction, character, and amount of growth, and their quantitative acid production in seven carbohydrate and related organic substances. Hemolysis and the gentian violet cultural test have been studied in part of the cultures.

We have not found that any of the features other than the fermentative reactions are sufficiently correlated with one another or with acid production to assist in the fixing of the organisms in groups or in distinguishing the source of isolation.

The seven organic substances tested show a definite order of

<sup>1</sup> *Bur. of Animal Ind. Bull.* 154, 1912.

availability for acid production. This order and the percentage of cultures yielding 1.2 per cent or more of acid when grown at 37° C. for three days is shown in the following table:

Glucose (monosaccharid) .....	98	per cent
Lactose (disaccharid) .....	76	per cent
Saccharose (disaccharid) .....	65.5	per cent
Salicin (glucosid) .....	42.7	per cent
Raffinose (trisaccharid) .....	37.5	per cent
Inulin (starch) .....	9.0	per cent
Mannite (an alcohol) .....	1.5	per cent

According to the positive reaction—over 1.2 per cent acid—in the test substances, 90 per cent of the cultures may be correlated so as to fall into nine groups.

Milk streptococci are distinguishable from the throat organisms of the same morphology (1) by their higher acid production in substances in which they grow, (2) by their greater independence of temperature relations, (3) by their general incapacity to ferment more complex test substances than the disaccharids. On the other hand, the throat strains in at least half the cases (1) ferment a more complex test substance and (2) in almost all cases fail to ferment a higher test substance than the monosaccharid at the room temperature.

We do not attempt to fix names or to establish any ultimate taxonomic relations by this study. We feel that the study, together with contemporary work in bacteriological biometrics, indicates the most fruitful, though laborious, way in which we are obliged to systematically split up our present unwieldy and meaningless genera.

In concluding, the authors wish to express their great appreciation of the hearty co-operation and skilful assistance of Marjorie H. Boyce, assistant in the Dublin Laboratory for 1912.

## A MOTILE CURVED ANAEROBIC BACILLUS IN UTERINE DISCHARGES.\*

ARTHUR H. CURTIS.

*(From the Memorial Institute for Infectious Diseases, Chicago.)*

In a bacteriological study of leukorrheal discharges now in progress, curved bacilli have been found in a considerable number of cases. These bacilli are as a rule sparsely scattered among other organisms without definite indication that they play a part in maintaining the leukorrhea.

Due to the small number of curved bacilli and the difficulty with which they are isolated, numerous early attempts to obtain pure cultures met with repeated failures. In two later cases, however, morphologically similar bacteria were found in large numbers. One patient suffered from an infection following instrumental abortion; in the other case infection complicated labor at full term. Smears from the first patient revealed an overwhelming number of gram-negative, curved bacilli. Stained specimens of the vaginal and cervical discharges obtained from the patient with puerperal infection contained a great preponderance of apparently identical organisms. There were also present scattered gram-negative, fusiform bacilli, a fair number of gram-positive diplococci, and some gram-negative diplococci.

A single set of cultures from the abortion case failed to yield the desired growth and material for later cultures was not available.

In anaerobic cultures from the vagina and from the uterus of the patient with puerperal infection, the bacillus was readily isolated.

### MORPHOLOGICAL CHARACTERISTICS.

The bacillus is a crescentically curved organism with rounded or tapering ends (Fig. 1). It equals the tubercle bacillus in length and is slightly greater in breadth. Staining occurs readily according to the usual methods. Gram's stain is not retained. There are no peculiarities of arrangement. Usually single, the bacillus some-

\* Received for publication February 6, 1913.



times occurs in twos, placed end to end. In such instances segments of a circle may be formed, or the concave surfaces may lie upon opposite sides, giving rise to an S-shaped arrangement.

#### MOTILITY.

In hanging-drop preparations, the bacillus is very actively motile. It moves rapidly across the field with a peculiar corkscrew motion. Possibly the result of exposure to air, diminution

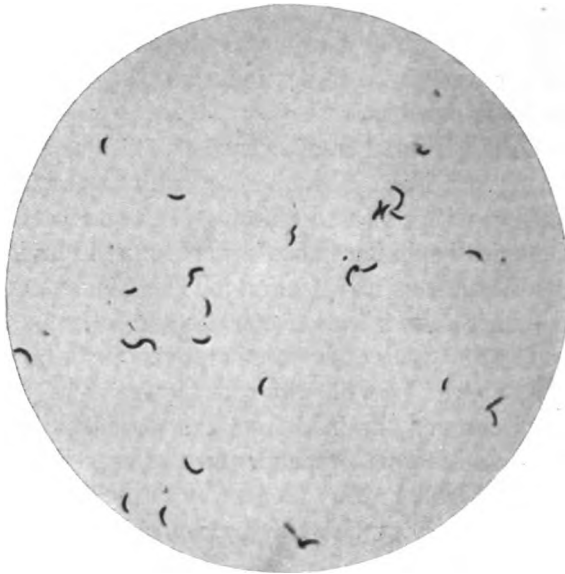


FIG. 1.—From culture on blood agar. Carbo gentian violet.  $\times 1,000$ .

in motility, accompanied by slight clumping, occurs within a few minutes.

Under dark field illumination no additional noteworthy characteristics are observed.

Flagella are present (see Fig. 2). A single organism may be provided with as many as half a dozen. The arrangement and point of attachment of the flagella is subject to much variation.

#### CULTURAL CHARACTERISTICS.

Anaerobic cultures on blood agar are scarcely visible at the end of 24 hours. In 48 hours is obtained a translucent, fairly good

growth. Growth occurs equally well on blood agar made from human, goat, or sheep blood. The culture medium is covered with a dustlike coat, composed of minute translucent colonies. In more dilute subcultures the colonies attain a diameter of two millimeters. Hemolysis does not occur. A somewhat disagreeable odor is noted upon opening the tubes. Anaerobic cultures on ascites agar develop only after a period of three days. The colonies attain pinhead size, are circular, elevated, and translucent.

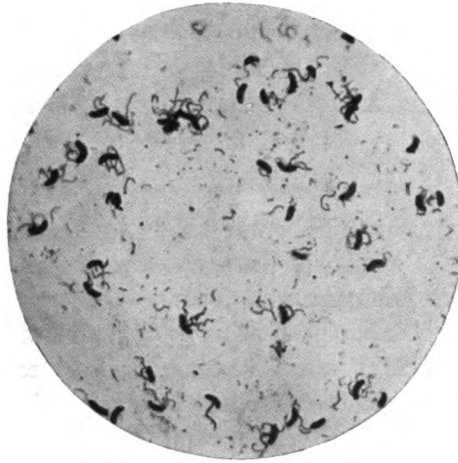


FIG. 2.—Flagella. Zettnow's silver nitrate method.  $\times 1,000$ .

The water of condensation is somewhat cloudy and contains many organisms.

Cultures made under anaerobic conditions on plain agar, broth, litmus milk, gelatin, and potato fail to develop. On alkaline agar some growth occurs. Organisms on this medium frequently show nodular or hook-shaped enlargements. On glucose agar occurs a slow, but good growth.

The bacillus is a strict anaerobe. Under partial exclusion of oxygen, development does not occur.

Optimum growth is attained at blood temperature.

Cultures grown at room temperature show very poor development at the end of one week.

Cultures developed in the incubator and thereafter maintained

at room temperature are viable at the end of four weeks. Heat at 55° C. for 15 minutes is sufficient to kill.

#### INJECTION OF RABBITS.

Cultures which had attained optimum growth were injected intravenously into rabbits without noticeable effect. Subcutaneous injection of two billions of bacilli was also harmless.

#### AGGLUTINATION.

Agglutination tests made during the height of the infection were weakly positive with the patient's serum, and negative with control serum. Similar tests performed during convalescence were negative.

#### CLASSIFICATION.

This anaerobe, invariably curved, is to be classed with the vibrios. In so far as can be ascertained a similar vibrio has not been cultivated. Lehmann and Neumann<sup>1</sup> in a consideration of vibrios and anaerobic bacilli mention no anaerobic vibrios or curved anaerobic bacilli. Menge and Kroenig<sup>2</sup> in a series of 70 vaginal examinations reported the frequent presence of comma-shaped bacilli. These were found in smears but could not be grown. Kroenig<sup>3</sup> obtained from the vagina an anaerobic streptococcus, non-pathogenic for animals, which was often found in symbiosis with a short curved bacillus. The curved bacillus lived for only one generation. Some of the curved organisms observed by Menge and Kroenig, and by Kroenig alone, are presumably identical with the one here described.

Heurlin<sup>4</sup> describes a curved gram-negative "*Bacillus circularis major*." His organism differs in that it produces hemolysis, takes anilin stains with the greatest difficulty, possesses neither motility nor flagella, and is pathogenic for animals.

The possibility of pelvic infection with this bacillus is to be

<sup>1</sup> *Bakt. Diagnostik*, V. Auflage, 1912.

<sup>2</sup> *Bakt. des weiblichen Genitalkanals*, 1895.

<sup>3</sup> *Centralbl. f. Gyn.*, 1895, 19, p. 400.

<sup>4</sup> *Bakt. Untersuchungen des Keimgehaltes im Genitalkanale*, etc., Helsingfors, 1910.

earnestly considered. Like the anaerobic *Streptococcus putridus* of Schottmüller, even though non-pathogenic for animals, it may be virulent for human subjects. The presence of the bacillus in overwhelming numbers in two cases of infection, together with the fact that similar organisms are found in patients with leukorrheal discharge, makes further clinical observation desirable.

## THE CONJUNCTIVAL REACTION FOR GLANDERS.\* (OPHTHALMIC TEST.)

K. F. MEYER.

(From the Laboratory of the Pennsylvania State Livestock Sanitary Board.)

During the last four years, in different parts of the world, extensive experiments have been conducted to find an accurate method by which glanders in horses can be quickly diagnosed by the practitioner without awaiting the result of one of the reliable serum tests. It has been found by Vallée,<sup>1</sup> Martell,<sup>2</sup> de Blicck,<sup>3</sup> Schnürer,<sup>4</sup> Müller, Gaehtgens and Aoki,<sup>5</sup> Fröhner,<sup>6</sup> Reinhardt,<sup>7</sup> Miessner,<sup>8</sup> and others that the local allergic reactions may be successfully used for this purpose, and that they undoubtedly offer great advantages and greater reliability than any of the other mallein tests. By reason of the fact that mallein, compared with tuberculin, gives a more pronounced local reaction when subcutaneously applied, theoretically, better results are expected in glanders with the local tests than in tuberculosis by similar methods. The investigations of Schnürer on more than 10,000 army horses were so encouraging that it was considered advisable to experiment along similar lines in Pennsylvania.

From preliminary experiments with mallein brute of the Institut Pasteur, and with our own preparations of similar composition, it was concluded that only a specially prepared standard mallein would promise uniformly satisfactory results. The assertion of Wladimiroff<sup>9</sup> that the use of an unknown, untested preparation

\* Received for publication January 2, 1913.

<sup>1</sup> *Bull. de la Soc. centr. de méd. vét.*, 1907, 61, p. 359.

<sup>2</sup> *Ibid.*

<sup>3</sup> *Ztschr. f. Infektionskrankh. usw. d. Haustiere*, 1910, 7, p. 418.

<sup>4</sup> *Ztschr. f. Infektionskrankh. usw. d. Haustiere*, 1908, 4, p. 216; *ibid.*, 1912, 10, pp. 321 and 408; *Deutsche tierarztl. Wchnschr.*, 1910, 18, p. 65.

<sup>5</sup> *Ztschr. f. Immunitätsf.*, 1911, 8, p. 626.

<sup>6</sup> *Monatschr. f. prakt. Tierh.*, 1912, 23, p. 1; *ibid.*, p. 433.

<sup>7</sup> *Monatschr. f. prakt. Tierh.*, 1911, 23, p. 178.

<sup>8</sup> *Centralbl. f. Bakteriöl.*, Abt. I, O., 1912, 63, p. 482.

<sup>9</sup> *Handbuch der Technik und Methodik der Immunitätsforschung*, Supplement I, 1911, p. 394; *ibid.*, 1908, 1, p. 1190.

will never permit comparative deductions, is only too true, and can without reserve be applied to many of the mallein products of commercial houses. All the ordinary "raw" mallein preparations are poor in the specific antigens and, particularly in occult cases of glanders, cause extremely weak and indistinct reactions. As none of the tested mallein preparations ("Mallein siccum Foth"; mallein of the "chemische Fabrik Humann und Teisler," etc.) used in Europe for the conjunctival tests were on the market in America, an attempt was made to prepare such a biologic product in our own laboratory. After several unsuccessful attempts, largely due to unsuitable glanders cultures, a mallein was obtained which gave unquestionably perfect results. The mallein used for this purpose is known under the name of "Mallein siccum Foth." Its preparation is given here, as it is of essential importance for the conjunctival test.

#### PREPARATION OF "MALLEIN SICCUH."

According to the outline given by Foth,<sup>1</sup> which practically has been followed in our laboratory, the preparation is as follows:

Slightly acid 2 per cent peptone and 4.5 per cent glycerin broth in wide Pasteur flasks (in the quantity of 250 c.c.) is inoculated with highly virulent glanders bacilli. The broth consists of equal parts of meat and potato extract. The glanders strain, selected for this purpose from a series of 20 strains from different outbreaks, has been passed through cats and tends to grow remarkably well on the surface of the broth. This feature is particularly important for the production of a potent mallein. For four to six weeks the cultures remain in the incubator at a temperature of 38° C. After being tested for purity and killed by heating to 70°-100° C., the fluid is concentrated slowly to  $\frac{1}{10}$  of its original volume in a vacuum distilling apparatus at a temperature of 75°-80° C. The syrup-like, brownish fluid is sucked through a number of folded filters. The process frequently is extremely slow, and the loss due to the filtration is comparatively high. The clear fluid is then precipitated in absolute alcohol (1 part of mallein to 30 parts of absolute alcohol). The pouring of the fluid into the alcohol should be done carefully, as otherwise a coarse, sticky deposit will result instead of a fine, flocculent, white-brownish one. The precipitation should be done only with absolutely water-free alcohol. The precipitate is filtered off, washed with absolute alcohol or ether, and spread on dry clay plates which are afterward put into an exsiccator and, under vacuum, dried over sulfuric acid. After 24 hours, a light, whitish powder can be scraped from the clay plates; this powder dissolves very readily in water, which it tinges a dark brown. A carefully prepared powder should not be hygroscopic, and, if protected from light (one of the main characteristics of mallein toxin is that it is very labile to light), will be active for one entire year and, according

<sup>1</sup> *Ztschr. f. Tiermedizin*, 1911, 15, p. 401; *Ueber die Gewinnung eines festen Malleins und über seine Bedeutung usw.*, Berlin, 1896.

to the experiences of others, probably longer. According to Foth, an old powder is more elective in its antigen properties than a fresh preparation, because the unspecific pyrogenous substances disintegrate more quickly than the specific mallein substances which in powder form remain practically unaltered.

#### TECHNIC OF THE REACTION.

Preliminary experiments have shown that the solution of the powder disintegrates rapidly, which is due in part to the absence of preservatives and in part to changes in toxin molecules. Therefore the powder form alone can be kept in stock, and the test solution should always be made shortly before use. It was found that the best and handiest equipment for the practicing veterinarian consists of two small bottles, one containing the powder, the other, sterile or saline water in such quantity as will make a 5 per cent solution of mallein. Bottles are kept in stock with the quantities which are necessary to test 10 or 50 horses respectively. It is considered that the quantity of 0.1 c.c. solution (according to the calculations made by Schnürer) is quite sufficient to test one horse. As explained, such prepared mallein deteriorates rapidly. Therefore the solution should be used immediately after its preparation and the remainder should be discarded. Recently, with a very strong mallein, weaker solutions of the powder (1 per cent and 2 per cent) have been tested and found to be as satisfactory as when a 5 per cent solution is used.

#### APPLICATION OF THE MALLEIN.

The solution of mallein is instilled by means of an eye-dropper in the quantity of two to three drops; it is not necessary to work absolutely quantitatively. During the last few months, in several glanders outbreaks, the mallein has been applied to the conjunctival sac by means of a camel-hair brush, with which, naturally, quantitative working is impossible. The eye should not be handled after the instillation has been made. A successful quantitative working is impossible because the animal usually closes its eyes after successful instillation, and a certain amount of the instilled mallein is expressed with the tears. The objection that the use of a camel-hair brush may act as an agent in the distribution of glanders has been disproved by experiments of Galtier and Schnürer. Instead of the eye-dropper or brush, Müller, Gaetgens, and Aoki have recommended the use of a glass rod. The camel-hair brush, which can be kept clean in carbolic acid when not in use, has the disadvantage of diluting the mallein solution with the conjunctival secretion. On the other hand, the application with the eye-dropper is, under ordinary circumstances, an extremely tedious task, particularly when testing young and nervous animals and when the light conditions in the stable are unfavorable. Special recommendations cannot be made and it is best to individualize according to the conditions. In any case, a careful examination of the eye in good light should be made before the mallein is applied. In one instance, a misleading result, similar to those discussed by Schnürer, was obtained in our experiments, largely due to the fact that an incipient conjunctivitis caused by a foreign body was overlooked at the time of inspection.

Schnürer frequently found an increase in temperature during the reaction and therefore advises the taking of temperatures in a manner that will be explained later. Miessner considers this rise unnecessary for a diagnosis and its detection too laborious for the benefit which can be derived from a temperature increase which is frequently only slight. Based on our own observations, the view of Miessner can, without

restriction, be supported. Only in four outbreaks previous to the application of the mallein, the temperatures were taken with "Reform" thermometers. I agree with Miessner that the temperature fluctuations are, in 98 per cent of the cases, so small that they can be recorded only when working with the greatest possible accuracy. Under the usual conditions, the results and their application for the diagnosis are, therefore, illusive and of no practical value.

#### REACTIONS.

In the majority of cases, shortly after the application of the mallein, slight lacrimation and perhaps an inferior degree of photophobia will be observed. For a few hours this reaction is absolutely non-specific and has nothing whatever to do with the specific reaction, which usually occurs five to seven hours after the application of the test. According to Schnürer, Fedders, and others, the specific reaction does not begin before the third hour. We did not observe any such early reaction. The specific reaction is, according to Schnürer's, Miessner's, and the writer's observations, characterized:

1. By moderate, profuse lacrimation, and pus-like, slimy secretion in the inner canthus of the eye. The degree varies considerably. Often there is only a small drop of pus; sometimes a real pyorrhea, extending in form of flakes over the entire orbital region or gumming the eyelids, and clinging to the hairs of the lids. The conjunctival sac is filled to a more or less pronounced degree with pus. The touching or the manipulation of the eyelids is in most cases extremely painful. The conjunctival membranes are deeply reddened, and in many instances there is a marked edema of the upper and lower lid, causing, therefore, a partial closure of the opening between the lids. Occasionally in such cases a glassy appearance of the mucous membranes with small petechiae is observed. The cornea and other portions of the eyeball were in none of our cases involved in the inflammatory process. Special attention was paid to these observations, as several Russian investigators (Krestowsky, Wladimiroff) claim to have observed turbidities of the aqueous humor. The inflammatory process is localized on the conjunctival membranes and is a suppurative conjunctivitis.

This reaction varies considerably in different animals and in the different stages of infection, and a scale of reactions can be noted



and should be reported as such. Only a discharge with leukocytes, that is to say, a purulent discharge, is to be considered as a positive reaction. There does not exist any uniformity of opinion as to this point of the reaction, which, in my opinion, is largely due to the fact that different mallein preparations have been used. All investigators (Schnürer, Miessner, and others), using the powdered mallein, agree on this point: *that only a discharge with pus can be considered a positive reaction.* In cases in which only a grayish, cigar-ash-like clump of a slimy discharge is found in the inner canthus, or in cases in which only a slight inflammatory reaction is noticeable, the reaction is regarded as questionable or doubtful, and marked (D); in all such cases, following the advice of Schnürer, a retest was made in 24 hours. The degree of pus in the discharge is marked P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>. To specify more clearly for the beginner, reading the ophthalmic reactions, it may be said that a conjunctivitis which can, as Schnürer states, be diagnosed at a distance should be marked P<sub>3</sub>. With this as a standard reaction, after a few practical observations, the beginner will have no difficulties in distinguishing the other degrees. During the last month, the conjunctival test has been applied in Pennsylvania by many veterinarians who were never personally instructed as to the interpretation of the reaction, and still the results, as checked by the serum tests, were uniformly good and often recorded with remarkable accuracy.

2. Schnürer, Miessner, and others have noted, contrary to the statement of de Blicke, that in 73 per cent of the horses which gave a positive ophthalmo-reaction 12 hours after the application of the ophthalmic mallein, a temperature rise of over 38.5° C. can be recorded. Schnürer emphasizes the great importance of this febrile reaction; Miessner, on the contrary, considers its recordance as not being worth the additional labor. In our cases, in about 80 per cent of the horses tested, the rectal temperature increased from 2° to 4° F. in the 18 hours following the application of the mallein, and decreased gradually toward the 24th hour. Naturally, only afebrile cases could be tested in these experiments. This temperature reaction takes place only in animals which are actually affected with glanders. Many comparative tests on a

fairly large number of healthy horses have shown that no temperature reaction takes place. A temperature reaction was noted only in those animals in which a marked ophthalmic reaction was present. The local reaction was, therefore, sufficiently distinct to constitute a diagnosis, and the tedious task of taking the temperatures with two thermometers for greater accuracy is, from a practical point of view, superfluous. Only in special cases do we use the thermic reactions as an additional criterion of the mallein test. In field work, taking the temperature can be omitted. In case temperatures are taken (advisable for scientific investigation), they should be recorded at intervals of four hours. Schnürer recommends the taking of two temperatures, namely, one at the time of application of the mallein, and one at the first reading, about 12 hours after the instillation.

#### DURATION OF THE REACTION.

Usually the reaction remains visible for 12-36 hours after the application of the biologic product. There is no absolute rule. Many cases have been observed in which the local reaction was present only during six hours following its first occurrence, and, on the other hand, cases have been recorded in which the reaction remained visible for 72 hours. These observations are in accordance with those of de Blicke, Wladimiroff, and others. We found it advisable to read the reactions from the eighth hour after the application of the mallein, as then, frequently, the early reactions are distinctly visible. A second reading should be made on the 12th-16th hour, and usually at this time the height of the reaction is present. If possible, a reading after 24 hours should also be taken. Schnürer recommends for the testing of the reactions the 12th and the 24th hour; Miessner, the 14th-20th hour after the application.

In our experience, so-called "atypical" (Schnürer) reactions occur not uncommonly; viz., the conjunctival test appears and disappears suddenly, or the reaction is distinct only after 24 hours. Several horses which were kept in our stable showed such reactions. They were mostly in the stage of acute infection.

Every precaution should of course be taken to avoid the possi-

bility that the purulent discharge is not washed off by the stable man or caretaker; it will rarely happen that the animals, by mutual licking, will remove the discharge. From our experience, it is better not to advise the caretaker of the horses to be tested as to the interpretation of the alterations in the eyes, as otherwise the veterinarian is apt to be deceived when making retests in the same stable. In all such cases a repetition is highly recommended. Perhaps the other eye is used for the test.

#### RETEST.

Schnürer has found that a second application of mallein will, in nearly all doubtful cases, produce a marked positive reaction, or become entirely negative. From our observations with the ophthalmic test in tuberculosis, we were quite familiar with this fact, that in a sensitized eye the reaction is more legible than in an unsensitized one. This hypersensitiveness of the conjunctival membrane as a sequel of the first application of mallein is, however, noted only in glandered but not in healthy animals. Schnürer's observations on 3,000, and our records on about 400 healthy horses have shown that a conjunctival test does not sensitize as long as the animal is not infected with glanders. *Several horses were retested three and four times and still no reaction was recorded.*

Differing from the tuberculin test, the *retest can be applied 24 hours after the first application*, a very decided advantage under present conditions of city veterinary sanitary police, where in large stables a final decision is immediately desired. In most cases in which the first reaction was doubtful, a distinct or negative result was seen after the second test. Still, in two horses, which probably were in the stage of incubation, no reaction was obtained in either test. All observations stand in correlation with similar ones of Miessner, Schnürer, etc.

In many stables, all horses, including reacting (occult) ones, were retested a third time after 14 days, for the purpose of detecting such animals as were in the stage of incubation at the first and second tests, and of excluding the possibility of a simulated reaction in the reacting horses. Only when this third conjunctival test again gave negative results were the horses considered to be free from glanders.

On making this third test, and in one stable after a fourth and a fifth test, we observed that the degree of reaction became less and less distinct. Only a slight conjunctivitis was noted in animals which at first gave classic reactions. *For these reasons more than three retests should not be applied.* The mallein probably does not cause accumulation of leukocytes, and therefore no inflammatory process, on account of the adaption of the cells to the biochemic changes or on account of absence of complement concentration.

In this connection another important question should be considered, namely, after what incubation time does a recently infected animal give a positive conjunctival reaction? Müller, Gaehtgens, Aoki, and Miessner have shown that the conjunctival reaction is visible from the fifth to ninth days in artificially infected animals. In one case of Müller, Gaehtgens, and Aoki, the reaction was not apparent until the 18th day after the infection. We have not conducted special experiments, as the epidemiologic observations seemed to prove the findings of the said investigators. Fröhner concluded from the observations of Müller, Gaehtgens, and Aoki that the ophthalmic test should be preferred to the agglutination and complement fixation tests on account of the earlier positive results recorded—positive evidence when the serum test is still negative. This conclusion did not meet the approval of Miessner. My observations lead me to coincide with Fröhner. One particular case is recorded herewith.

A horse standing close to another which showed distinct glanders lesions on autopsy, developed a characteristic nasal discharge four days after it was removed from the infected stable (8–10 days after probable infection). The serum tests the first day were: agglutination 1:100; complement fixation, negative; the third day a marked ophthalmic reaction was noted; the fourth day, agglutination 1:500; complement fixation, negative. Based on the ophthalmic test, the horse was killed and glanders of the nasal septum, submaxillary lymph glands, and a few very small nodules in the lungs were demonstrated.

In a few other cases the ophthalmic test indicated distinctly the presence of a glanders infection at a time when the serum test did not indicate the slightest sign of a production of antibodies.

This occurred when, as was shown by autopsy, the glanders infection was slight and caused by *B. mallei* of low virulence (experiments). In these cases the production of agglutinins took place extremely slowly and over a long period of time, as in the cases recorded by Bonome. The conjunctival test, however, gave distinct reactions and allowed an early diagnosis.

In our opinion the conjunctival test is reliable for the determination of recently infected cases and certainly is, at this stage, frequently preferable to the serum tests (see p. 185).

#### CORRELATION OF THE CONJUNCTIVAL TEST TO THE SUBCUTANEOUS MALLEIN TEST.

The application of the subcutaneous mallein test is legally required in Pennsylvania and, therefore, the influence of this test on the conjunctival test was the subject of different observations. It is a well known fact that the subcutaneous application of mallein often suppresses somewhat the intensity of the local reactions, but this effect is, according to Foth, not marked nor of great practical importance. Many horses were tested by the subcutaneous method and afterward by the ophthalmic method and in no instance was a reaction noted when the horse was not infected with glanders. The ophthalmic test in most of the experimental cases followed the subcutaneous one in the next 24 hours. In several instances, 10-14 days elapsed before an ophthalmic test was made, and still no reaction was noted. In our experience the subcutaneous test does not influence a subsequent conjunctival test applied in the next 24 hours; a point which may be of great assistance to the practitioner. In an easy manner a doubtful temperature reaction can be checked by the ophthalmic test. As temporary febrile reactions are not uncommon in healthy draft horses, and as the failures of the subcutaneous mallein test in healthy animals, according to my statistical investigations, exceed 14 per cent, a simple test like the ophthalmic test will certainly be welcomed.

The subcutaneous mallein test, on the other hand, is not influenced by a preceding ophthalmic test. In case the conjunctival test was positive, a subcutaneous test applied during the following 3-14 days, causes a reappearance of the eye reaction often to a

much more distinct degree than at the time of the ophthalmic test. Animals in which such reactions occur are unquestionably affected with glanders; Schnürer says that the reappearance of past inflammatory symptoms of the conjunctiva during a subcutaneous malleinization is an absolutely conclusive proof of a glanders infection. Such conditions were frequently observed during our tests.

The simultaneous application of a subcutaneous and conjunctival test should be avoided, as in many animals during the febrile reaction a sudden fading away of the existing conjunctival reaction or a late appearance of it (after the fever has decreased) will be noted. The conditions are similar to those observed in tuberculosis (see Foth and others).

From our observations, we recommend the application of the conjunctival test first and, if necessary, the subcutaneous test in the second place and not in the reversed succession.

#### RESULTS OBTAINED AND GENERAL CONCLUSIONS.

In considering all the details mentioned, 210 horses were tested, from April until July. Since that date, about 400 additional horses were tested, checked by the serum tests and subsequent retests, but, as far as the autopsies and conjunctival tests are concerned, were not under my supervision. In the following table, therefore, are included only my own observations on 210 horses. The additional tests were as satisfactory as the experimental tests, and the deductions can, therefore, be applied to about 600 horses.

All our examinations as to the worth of the conjunctival reaction were conducted in conjunction with the laboratory tests, particularly the complement fixation and agglutination tests. The readings of the conjunctival reaction were made by the writer or by a trained assistant or agent of the Pennsylvania State Livestock Sanitary Board. The blood for the serum tests was collected before the mallein was instilled. The serum tests were all conducted by my first assistant under personal supervision in the laboratory, as usual and in the manner described in a recent publication. The autopsies of the animals found to be affected with glanders were conducted either in the postmortem room of the School of Veterinary Medicine, or in the field, in the presence of the writer. In

No.	CLINICAL SYMPTOMS	SERUM TEST I		MALLEIN SUBCUT. MAX. TRM.	OPH-THALMIC TEST I	SERUM TEST II		OPH-THALMIC TEST II	AUTOPSY
		Complement Fixation Test	Agglutination Test			Complement Fixation Test	Agglutination Test		
1	.....	0.05	1:800	.....	P <sub>3</sub>	.....	.....	.....	N., L., and Lgl.
2	.....	.....	1:500	.....	N	.....	.....	.....	.....
3	.....	0.1 influence by mallein	1:1000	.....	N	.....	1:600	.....	.....
4	.....	0.1	1:400	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
5	.....	.....	1:800	.....	N	.....	.....	.....	.....
6	6-45 horses of one stable.....	.....	1:200	103	N	.....	.....	.....	.....
7	.....	.....	1:200	105	N	.....	.....	.....	.....
8	.....	.....	1:200	103.3	N	.....	.....	.....	.....
9	.....	.....	1:100	103.2	N	.....	.....	.....	.....
10	.....	.....	1:100	104.2	N	.....	.....	.....	.....
11	.....	0.02	1:2000	103.1	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
12	.....	.....	1:500	105.4	N	.....	.....	.....	.....
13	.....	0.05	1:800	105.5	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
14	.....	0.1	1:1000	104.4	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
15	.....	.....	1:800	105.5	N	.....	.....	.....	.....
16	.....	0.1	1:400	105	P <sub>1</sub>	.....	.....	.....	L. and Lgl.
17	.....	.....	1:300	103.2	N	.....	.....	.....	.....
18	.....	.....	1:400	103.3	N	.....	.....	.....	.....
19	.....	.....	1:200	103.2	N	.....	.....	.....	.....
20	.....	.....	1:100	104.2	N	.....	.....	.....	.....
21	.....	.....	1:200	104.2	N	.....	.....	.....	.....
22	.....	.....	1:200	103.5	N	.....	.....	.....	.....
23	.....	.....	1:300	104	N	.....	.....	.....	.....
24	.....	.....	1:400	103.1	N	.....	.....	.....	.....
25	.....	.....	1:300	106	N	.....	.....	.....	.....
26	.....	.....	1:400	102	N	.....	.....	.....	.....
27	.....	.....	1:600	101.2	N	.....	.....	.....	.....
28	.....	.....	1:300	102.2	N	.....	.....	.....	.....
29	.....	.....	1:200	102	N	.....	.....	.....	.....
30	.....	.....	1:200	102.1	N	.....	.....	.....	.....
31	.....	.....	1:400	103.0	N	.....	.....	.....	.....
32	.....	.....	1:400	101.2	N	.....	.....	.....	.....
33	.....	.....	1:500	104.3	N	.....	.....	.....	.....
34	.....	.....	1:400	103.2	N	.....	.....	.....	.....
35	.....	.....	1:100	102	N	.....	.....	.....	.....
36	.....	.....	1:200	104.2	N	.....	.....	.....	.....
37	.....	.....	1:200	102	N	.....	.....	.....	.....
38	.....	.....	1:600	103	N	.....	.....	.....	.....
39	.....	.....	1:400	101.3	N	.....	.....	.....	.....
40	.....	.....	1:100	102.3	N	.....	.....	.....	.....
41	.....	.....	1:100	101	N	.....	.....	.....	.....
42	.....	.....	1:200	100.2	N	.....	.....	.....	.....
43	.....	.....	1:400	103	N	.....	.....	.....	.....
44	.....	.....	1:300	102.2	N	.....	.....	.....	.....
45	.....	.....	1:200	100.4	N	.....	.....	.....	.....
46	46-84 horses of one outbreak.	.....	1:200	102	N	.....	.....	.....	.....
47	.....	.....	1:300	102	N	.....	.....	.....	.....
48	.....	.....	1:500	103	N	.....	.....	.....	.....
49	.....	.....	1:400	104	N	.....	.....	.....	.....
50	.....	.....	1:300	103.3	N	.....	.....	.....	.....
51	.....	.....	1:200	102.3	N	.....	.....	.....	.....
52	.....	.....	1:100	104	N	.....	.....	.....	.....
53	.....	.....	1:200	101	N	.....	.....	.....	.....
54	.....	.....	1:400	104	N	.....	.....	.....	.....
55	.....	.....	1:300	102	N	.....	.....	.....	.....
56	.....	.....	1:200	102.2	N	.....	.....	.....	.....
57	.....	.....	1:200	101	N	.....	.....	.....	.....
58	.....	.....	1:100	103.3	N	.....	.....	.....	.....
59	.....	.....	1:200	101.4	N	.....	.....	.....	.....
60	.....	.....	1:400	101	N	.....	.....	.....	.....
61	.....	.....	1:300	102.2	N	.....	.....	.....	.....

The horses of one and the same outbreak are indicated accordingly.

N. = nasal passages.

L. = lungs.

Sk. = skin.

Lgl. = lymph glands.

No.	CLINICAL SYMPTOMS	SERUM TEST I		MALLEIN SUBCUT. MAX. TEM.	OPH-THALMIC TEST I	SERUM TEST II		OPH-THALMIC TEST II	AUTOPSY
		Complement Fixation Test	Agglutination Test			Complement Fixation Test	Agglutination Test		
62	.....	.....	1:300	102	N	.....	.....	.....	.....
63	.....	.....	1:200	104.2	N	.....	.....	.....	.....
64	.....	.....	1:400	104	N	.....	.....	.....	.....
65	.....	.....	1:200	.....	N	.....	.....	.....	.....
66	.....	.....	1:500	.....	N	.....	.....	.....	.....
67	.....	.....	1:200	.....	N	.....	.....	.....	.....
68	.....	0.05	1:1500	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
69	Farcy, nasal discharge	0.02	1:1000	pos.	P <sub>3</sub>	.....	.....	.....	N. and L.
70	.....	0.05	1:1500	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
71	.....	.....	1:200	100	N	.....	.....	.....	.....
72	.....	.....	1:400	100.2	N	.....	.....	.....	.....
73	.....	.....	1:200	100.1	N	.....	.....	.....	.....
74	.....	.....	1:100	100.2	N	.....	.....	.....	.....
75	.....	0.05	1:1000	.....	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
76	.....	0.05	1:2000	.....	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
77	.....	0.02	1:1000	.....	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
78	Nasal discharge...	0.1	1:2000	105.4	P <sub>3</sub>	.....	.....	.....	Acute infection N. and L.
79	Nasal discharge...	0.1	1:1000	103.4	P <sub>3</sub>	.....	.....	.....	Acute infection N. and L.
80	.....	.....	1:300	.....	N	.....	.....	.....	.....
81	.....	.....	1:300	.....	N	.....	.....	.....	.....
82	.....	.....	1:500	.....	N	.....	.....	.....	.....
83	Farcy, nasal discharge	0.05	1:1000	.....	P <sub>3</sub>	.....	.....	.....	Culture obtained
84	Farcy, nasal discharge	0.05	1:2000	.....	P <sub>2</sub>	.....	.....	.....	Not made Culture obtained
85	.....	0.1	1:1000	pos.	P <sub>4</sub>	.....	.....	.....	N., L., and Lgl.
86	.....	.....	1:100	neg.	N	.....	.....	.....	.....
87	Nasal discharge...	.....	1:200	.....	N	.....	.....	.....	.....
88	Lesions on skin	.....	1:400	.....	N	.....	.....	.....	.....
89	.....	.....	1:200	.....	N	.....	.....	.....	.....
90	.....	.....	1:400	.....	N	.....	.....	.....	.....
91	.....	.....	1:300	.....	N	.....	.....	.....	.....
92	Nasal discharge...	0.02	1:1000	.....	P <sub>2</sub>	.....	.....	.....	N. and L.
93	Nasal discharge...	0.02	1:800	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
94	Nasal discharge...	0.05	1:600	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
95	.....	0.05	1:600	.....	N	.....	.....	.....	.....
96	.....	0.05	1:1500	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
97	.....	.....	1:800	.....	Tem. 102.3	.....	.....	.....	.....
98	Nasal discharge...	0.05	1:600	.....	N	.....	.....	.....	.....
99	.....	.....	1:200	.....	N	.....	.....	.....	.....
100	.....	.....	1:500	.....	N	.....	1:500	N	.....
101	.....	.....	1:1000	.....	P <sub>3</sub>	0.1	1:1000	P <sub>3</sub>	N., L., and Lgl.
102	.....	.....	1:1000	.....	P <sub>3</sub>	0.1	1:1000	P <sub>3</sub>	N., L., and Lgl.
103	.....	.....	1:1000	.....	P <sub>3</sub>	0.1	1:1000	P <sub>3</sub>	N., L., and Lgl.
104	.....	.....	1:600	.....	N	.....	.....	.....	.....
105	.....	.....	1:400	.....	N	.....	.....	.....	.....
106	.....	.....	1:400	.....	N	.....	.....	.....	.....
107	.....	.....	1:600	.....	N	.....	.....	.....	.....
108	Nasal discharge...	0.05	1:1500	.....	P <sub>3</sub>	.....	.....	.....	N. and L.
109	100-145 horses of one outbreak	.....	.....	.....	N	.....	1:100	N	.....



No.	CLINICAL SYMPTOMS	SERUM TEST I		MALLEIN SUBCUT. MAX. TEM.	OPH-THALMIC TEST I	SERUM TEST II		OPH-THALMIC TEST II	AUTOPSY
		Complement Fixation Test	Agglutination Test			Complement Fixation Test	Agglutination Test		
110	.....	.....	.....	.....	P <sub>3</sub>	0.05	1:800	P <sub>1</sub>	L. and Lgl.
111	.....	.....	.....	.....	.....	.....	1:200	N	.....
112	.....	.....	.....	.....	N	.....	1:100	N	.....
113	.....	.....	.....	102.2	N	0.1 M	1:100	N	.....
114	.....	.....	.....	{ 101.4 }	N	.....	1:200	N	.....
115	.....	.....	.....	{ 101.4 }	P <sub>2</sub>	0.05	1:200	P <sub>3</sub>	L. and Lgl.
				{ 104.8 }	.....	.....	.....	4 days on re-test	
116	.....	.....	.....	.....	N	.....	1:300	N	.....
117	.....	.....	.....	.....	.....	.....	1:200	N	.....
118	.....	.....	.....	.....	.....	.....	1:100	N	.....
119	.....	.....	.....	.....	.....	.....	1:500	N	.....
120	.....	.....	.....	.....	.....	.....	1:200	N	.....
121	.....	.....	.....	101.8	N	0.1 M	1:200	N	.....
122	.....	.....	.....	.....	N	.....	1:200	N	.....
123	.....	.....	.....	103.4	P <sub>1</sub>	0.05	1:300	P <sub>3</sub>	L. and Lgl.
124	.....	.....	.....	103.4	P <sub>2</sub>	0.05	1:200	P <sub>2</sub>	L. and Lgl.
				(104.9)	.....	.....	.....	.....	
125	.....	.....	.....	100.5	N	0.05 M	1:500	N	.....
126	.....	.....	.....	100.4	N	0.1 M	1:100	N	.....
127	.....	.....	.....	.....	N	.....	1:400	N	.....
128	.....	.....	.....	.....	P <sub>2</sub>	0.2 P	1:200	P <sub>1</sub>	L. and Lgl.
				104.4	(105.2)	.....	.....	.....	
129	.....	.....	.....	(104.8)	P <sub>3</sub>	0.2 P	1:200	P <sub>3</sub>	L. and Lgl.
				105.2	(104.4)	.....	.....	.....	
130	.....	.....	.....	105.3	N	.....	1:100	N	.....
131	.....	.....	.....	.....	N	.....	1:500	N	.....
132	.....	.....	.....	103	P <sub>3</sub>	0.4 partial	1:1500	P <sub>1</sub>	L. and Lgl.
				.....	.....	0.05	.....	(P <sub>3</sub> )	
133	.....	.....	.....	105.4	P <sub>3</sub>	.....	1:500	P <sub>2</sub>	L. and Lgl.
				.....	(103.8)	.....	.....	.....	
134	.....	.....	.....	100	.....	.....	1:100	N	.....
135	.....	.....	.....	101	P <sub>1</sub>	0.05	1:100	.....	L. and Lgl.
				.....	(101.4)	.....	.....	.....	
136	.....	.....	.....	.....	N	.....	1:100	N	.....
137	.....	.....	.....	.....	N	.....	1:200	N	.....
138	.....	.....	.....	.....	N	.....	1:400	N	.....
139	.....	.....	.....	.....	N	.....	1:100	.....	.....
140	.....	.....	.....	.....	N	.....	1:200	N	.....
141	.....	.....	.....	.....	N	.....	1:500	N	.....
142	.....	.....	.....	104.4	P <sub>2</sub>	0.1	1:200	N	L. and Lgl.
				(103.6)	(103.5)	.....	.....	.....	
143	.....	.....	.....	.....	.....	.....	1:300	N	.....
144	.....	.....	.....	.....	.....	.....	1:200	N	.....
145	.....	.....	.....	.....	.....	.....	1:200	N	.....
146	146-166 horses of one out-break	.....	1:100	100.0	.....	.....	.....	.....	.....
147	Serum test only applied to remainder of animals	.....	.....	.....	.....	.....	.....	.....	.....
148	.....	.....	1:100	102.2	N	.....	.....	.....	.....
149	.....	.....	1:200	101	N	.....	.....	.....	.....
150	.....	.....	1:200	103	N	.....	.....	.....	.....
151	.....	.....	1:100	105.4	P <sub>2</sub>	.....	.....	.....	L. and Lgl.
152	.....	.....	1:200	102.2	N	.....	.....	.....	.....
153	.....	.....	1:200	103.4	N	.....	.....	.....	L. and Lgl.
154	.....	.....	1:100	105.8	P <sub>3</sub>	.....	.....	.....	.....
155	.....	.....	1:100	102.8	N	.....	.....	.....	.....
156	.....	.....	1:100	100	N	.....	.....	.....	.....
157	.....	.....	1:200	100	N	.....	.....	.....	.....
158	.....	.....	1:100	102	N	.....	.....	.....	.....
159	.....	.....	1:200	104.4	N	.....	.....	.....	.....
160	.....	.....	.....	103	P <sub>3</sub>	.....	.....	.....	L. and Lgl., Sk.

No.	CLINICAL SYMPTOMS	SERUM TEST I		MALLEIN SUBCUT. MAX. TEM.	OPH-THALMIC TEST I	SERUM TEST II		OPH-THALMIC TEST II	AUTOPSY
		Complement Fixation Test	Agglutination Test			Complement Fixation Test	Agglutination Test		
161	.....	.....	1:200	105.4	N	.....	.....	.....	.....
162	.....	.....	1:100	105.6	N	.....	.....	.....	.....
163	.....	.....	1:200	103.2	N	.....	.....	.....	.....
164	.....	.....	1:100	102	N	.....	.....	.....	.....
165	.....	.....	1:200	101.2	N	.....	.....	.....	.....
166	.....	.....	1:100	102.2	N	.....	.....	.....	.....
167	.....	.....	.....	104.6	P <sub>3</sub>	.....	.....	.....	Sk., L., and Lgl.
168	.....	.....	.....	104	P <sub>1</sub>	0.02	1:200	.....	L. and Lgl.
169	.....	.....	.....	105	P <sub>1</sub>	0.02	1:500	.....	L. and Lgl.
170	.....	.....	.....	104.4	P <sub>3</sub>	.....	.....	.....	L. and Lgl.
171	.....	.....	.....	104.2	P <sub>2</sub>	.....	.....	.....	Sk., L., and Lgl.
172	.....	0.05	1:1500	.....	P <sub>2</sub>	.....	.....	.....	Sk., L., and Lgl.
173	.....	.....	1:200	.....	N	.....	.....	.....	.....
174	.....	.....	1:200	.....	N	.....	.....	.....	.....
175	.....	.....	1:500	.....	N	.....	.....	.....	.....
176	.....	.....	1:200	.....	N	.....	.....	.....	.....
177	.....	.....	1:100	.....	N	.....	.....	.....	.....
178	.....	.....	1:200	.....	N	.....	.....	.....	.....
179	.....	.....	1:200	.....	P <sub>2</sub>	0.05	1:1000	P <sub>2</sub>	Isol.
180	.....	.....	1:100	.....	N	.....	1:100	.....	.....
181	.....	0.02	1:2000	.....	P <sub>2</sub>	.....	.....	.....	Sk. and L.
182	.....	0.01	1:4000	.....	P <sub>1</sub>	.....	.....	.....	Sk. and L.
183	.....	.....	1:400	101.5	N	.....	.....	.....	.....
184	.....	0.3	1:1000	104.5	P <sub>3</sub>	.....	.....	.....	Sk. and L.
185	.....	.....	1:100	103.6	N	.....	.....	.....	.....
186	.....	.....	1:200	.....	N	.....	.....	.....	Sk., L., and Lgl.
187	.....	0.01	1:4000	.....	P <sub>3</sub> (48 after applica- tion)	.....	.....	.....	Sk. and L.
188	.....	0.02	1:1000	.....	P <sub>3</sub>	.....	.....	.....	Sk., L., and Lgl.
189	Nasal discharge...	0.02	1:1500	.....	P <sub>3</sub>	.....	.....	.....	L. and Lgl. and N.
190	Nasal discharge...	0.01	1:4000	.....	P <sub>3</sub>	.....	.....	.....	L. and Lgl. N.
191	.....	0.2	1:1000	.....	P <sub>1</sub>	.....	.....	.....	L. and Lgl.
192	Nasal discharge...	0.1	1:1500	.....	P <sub>3</sub>	.....	.....	.....	L. and N.
193	.....	.....	1:400	.....	N	.....	.....	.....	.....
194	Discharge from both nostrils	0.1	1:800	.....	P <sub>4</sub>	.....	.....	.....	N. and L.
195	195-208 horses of one outbreak	.....	.....	.....	.....	.....	.....	.....	.....
196	.....	.....	.....	100.2	N	.....	1:200	.....	.....
197	.....	.....	.....	103.1	N	.....	1:500	.....	.....
198	.....	.....	.....	100	N	.....	1:200	.....	.....
199	.....	.....	.....	100.2	N	.....	1:500	.....	.....
200	.....	.....	.....	100.2	N	.....	1:500	.....	.....
201	.....	.....	.....	103.1	N	.....	1:300	.....	.....
202	.....	.....	.....	100.3	N	.....	1:400	.....	.....
203	.....	.....	.....	101.2	N	.....	1:200	.....	.....
204	.....	.....	.....	100.2	N	.....	1:300	.....	.....
205	.....	.....	.....	100.4	N	.....	1:600	.....	.....
206	.....	.....	.....	100	N	.....	1:200	.....	.....
207	.....	.....	.....	100.2	N	.....	1:500	.....	N., Sk., L., and Lgl.
208	.....	.....	.....	104.8	P <sub>4</sub>	(0.02)	1:1000	.....	.....
209	.....	.....	.....	103	N	0.05	1:800	.....	L. and Lgl.
210	.....	.....	.....	.....	N	.....	1:100	.....	.....
210	.....	.....	.....	.....	N	.....	1:200	.....	.....

a few instances where clinical cases were diagnosed, a detailed autopsy was omitted when the *B. mallei* had been isolated from the organs or secretions submitted for examination. As quite a few veterinarians still consider the Strauss reaction to be the chief diagnostic method for glanders, material is frequently submitted for laboratory diagnosis without our request, and has been successfully used for the inoculations mentioned before a test was applied. In rare cases the animals were tested only by the ophthalmic method and were killed before the serum tests were concluded. In such cases the autopsies were made with particular care and, if necessary, animals were inoculated. The material was not especially selected but came from different outbreaks of glanders which came to the notice of the Pennsylvania State Livestock Sanitary Board. In most instances the animals tested did not show any clinical symptoms (occult cases of glanders) and thus gave the conjunctival test a more severe trial.

The results are given in the table, which shows that of 210 horses, 58 were found by means of the complement fixation test to be suffering from glanders. The interpretation of this test is based on the principle as outlined by Miessner and others and shown in my publication on this subject. Statistical investigations (see publications from 1909-12) show that 99.6 per cent correct results in glanders and 99.75 per cent in healthy horses are obtained with the complement fixation test. Of the 58 glanders cases, only 56 reacted positively to the conjunctival test, while two horses which proved at postmortem to be affected with glanders did not give any reaction whatever. On account of the positive serum reactions, both animals were condemned and, therefore, a third retest at 14 days' interval could not be carried out. The two animals, Nos. 206 and 208, were, according to the history and the result of the serum tests, in the stage of incubation and would probably have shown a positive reaction on a third retest. In the experimental horse No. 28 conditions observed by Müller, Gaehtgens, and Aoki were therefore existing, namely, *in quite recent infections the conjunctival tests may be negative and occur only several days after the appearance of the antibodies in the serum of the patient.* Whether or not this is an exception has to be determined by further

observations. We found lately that these conditions are rare (see also p. 178).

The retest, 14 days after the first test, gave, with a few exceptions, distinct results. Miessner concluded from his observations that in many cases, probably on account of a certain adaptation (also in glanders horses), only a slight reaction will be noted. In his opinion, only the complement fixation test should be used for a retest in a stable. In our opinion, the second serum test can very readily be combined with a third ophthalmic test.

As previously explained, the subcutaneous mallein test (legal requirement) was applied several times at 14-day intervals. The horses (for example, No. 115 and No. 135) became gradually used to the mallein and did not show any febrile reactions, and yet the ophthalmic mallein test was, in all instances, positive. This fact is of great importance, particularly when the serum tests, on account of a previous mallein application, are misleading. In horses that are maliciously injected with mallein to veil the results of a subsequent test by a state official, the conjunctival test will be of great assistance in disclosing the true condition. So far as we know, the use of antipyretics to falsify the mallein test is not commonly practiced, and the results of the ophthalmic test under these conditions have to be determined. Experiments for this purpose are in progress. In what manner the mallein vaccine used in New York City influences the conjunctival test has also to be determined. Practical experience has shown that the serum tests are misleading (over a long period agglutinins and complement-fixing antibodies are present).

In other diseases, particularly sporotrichosis, with all its clinical similarities to glanders, a positive reaction to the ophthalmic test was never obtained.

To give a few examples of the readings of the reactions, two characteristic cases are selected. The limited space at our disposal will not permit the giving of all the readings in detail as has been done in other publications (Schnürer, Fröhner, Reinhardt, Miessner, and others).

*No. 1:* Horse, bay gelding; temperature 100.8° F. at 9 P.M. Showed 10-5-12, 10 hours after the application of the ophthalmic mallein, severe lacrimation, photophobia, profuse purulent discharge, temperature 104.8.

*Serum test.*—Complement fixation, 0.05 binding value, agglutination 1:200. The animal was retested 29-7-12 and showed for three days a severe purulent discharge, lacrimation, and photophobia. Complement fixation 0.05, agglutination 1:400. This animal was killed and the autopsy showed four old glanders foci in lungs and two large cheesy glanders nodules in the bronchial lymph glands.

*No. 2:* Horse, bay, 10 years old, weight 1,300 lbs., condition very good; submaxillary lymph glands, slightly hardened; few small nodules 10 inches above the haunch. Ophthalmic mallein applied 8 P.M. First observation 8 A.M., 8-10-12, eye showed slight lacrimation and photophobia. At the inner canthus of the eye was a very small amount of whitish slimy material. Second test applied 8-10-12, 10 P.M. First observations 8 A.M., 8-11-12. Excessive lacrimation, pronounced photophobia, edematous swelling of the lids, and considerable amount of whitish yellow pus-like discharge at the inner canthus. Complement fixation 0.02; agglutination 1:1500.

*Postmortem examination.*—Glanders of the lungs, the bronchial and submaxillary lymph glands and integumentum.

In the table is also shown that not one of the healthy horses (152) gave a positive ophthalmic reaction. That these horses were really not affected with glanders was proven primarily by successive serum tests and a careful observation and control during the last five months of all the stables in which the test was applied. No further cases of glanders have developed and the disease can, therefore, be considered as having been eradicated. In several of the stables, only recently serum tests were conducted and not one of the animals has reacted. The results obtained in the healthy horses correspond with those mentioned by Reinhardt, Wladimiroff, Klimmer,<sup>1</sup> Dedjulin,<sup>2</sup> Miessner, and others; 100 per cent correct results were obtained by these investigators. Schnürer reports 22.3 per cent failures in testing 5,450 animals. These results are based upon reports which were submitted by veterinarians, many of whom had not the training necessary to interpret the reactions. Such mistakes will undoubtedly be eliminated in the future when the method has become perfectly familiar to the profession. In apparently healthy horses a reaction to the conjunctival test followed by failure to demonstrate anatomical lesions certainly should not be considered as proof of the inefficiency of this test. Everyone familiar with the results of the tuberculin test will be prepared to admit that also in glanders similar conditions occasionally prevail, and that only a most care-

<sup>1</sup> *Handbuch der Serum-Therapie und Serum-Diagnostik in der Veterinär Medizin*, 1911, p. 321.

<sup>2</sup> *Ztschr. f. Infektionskrankh. usw. d. Haustiere*, 1912, 15, p. 365.

ful autopsy will reveal minute anatomical lesions. As autopsies are not very agreeable to the veterinarian under usual conditions, the number of failures reported out of a large number of animals tested will naturally be greater than are recorded in the few tests in this paper.

All in all, the conjunctival method certainly cannot be blamed for these failures, and, compared with the subcutaneous mallein test, the results obtained are remarkably accurate. In addition, the simple manner of application, the relief from time-absorbing taking of temperatures with all its disadvantages, will certainly place this test in the first rank of the diagnostic methods for glanders.

#### THE RESULTS COMPARED WITH THE COMPLEMENT FIXATION AND AGGLUTINATION TESTS.

In the interest of a perfect sanitary control, a centralized record system of all glanders cases must be maintained. To enforce such a legalized system it is necessary that the diagnosis of glanders be established independently of the practitioner, especially as the state is giving compensation for the destruction of the animals. The checking of the field tests for glanders and the confirmation of the diagnosis is most efficiently done in the state of Pennsylvania by the serum tests.

In the table, the results are shown and compared with similar observations of Dedjulin and Miessner. We have noted the very satisfactory and remarkably accurate results with the complement fixation test. In a recent publication special emphasis has been paid to this fact.<sup>1</sup>

As mentioned before, in the state of Pennsylvania the use of the subcutaneous mallein test is still required. It was therefore not surprising to find that several sera in our tests gave positive reactions in the complement fixation test when there was no sign whatever of glanders, and when the retest proved that such animals were not suffering from the disease. We had 3.2 per cent failures in the healthy horses and attribute this fact to the production of immune bodies by previous injections of mallein. *We earnestly*

<sup>1</sup> K. F. Meyer, *Proc. of the Amer. Vet. Med. Assn.*, 1912.

*request, therefore, that the proper authorities arrange that when a serum test is to be applied, no subcutaneous mallein test be made; a request which is reasonable on account of the facts explained, and which has been recognized as proper procedure in Prussia (for the last four years).* The veterinary sanitary laws of Prussia forbid the use of mallein (subcutaneously) on account of its effect on the interpretation of the serum tests.

The agglutination test, in considering 1:800 a reaction, showed rather unfavorable results, as by it alone only 74.5 per cent of the actual cases would have been detected. By it 2.6 per cent of the healthy horses gave a positive reaction, and might have been considered as suffering from glanders. The agglutination test alone would therefore have been very unreliable. This observation has been critically discussed in my last publication. It is impossible to mark a certain *limes titre* as an indication of a positive reaction. Only reactions of 1:2000 and above this titre can be considered as conclusive. Since the introduction of the complement fixation test, we use the agglutination test as a control only for the period of incubation, and never has a diagnosis been made based solely on the result of the agglutination test. We will retain the agglutination test as a laboratory method and not follow the proposal of Miessner to replace the agglutination test by the conjunctival test. These conclusions stand in correlation with those of Schnürer, Fröhner, Reinhardt, and others.

A few observations were made as to the effect of the ophthalmic mallein on the serum reactions. In no instance was an increase of the agglutinins and complement-fixing antibodies noted. The observations of Miessner made on glandered horses are not conclusive, as animals were selected in which the increase of the antibodies as a natural sequel of the infection was to be expected.

#### CONCLUSIONS AND RECOMMENDATIONS.

In considering these investigations, the following conclusions can be drawn:

1. The conjunctival test for glanders is very reliable. It can, in a short time, without large expense, be applied by every prac-

ticing veterinarian and will permit the untrained to make a diagnosis of glanders with the greatest possible accuracy.

2. The serum tests are necessary to centralize the control of infectious diseases in a reliable state institution and to support the diagnosis in case compensation is sought by the owner of the animal. Only the complement fixation test can be used independently for the diagnosis of glanders.

In fulfilling these requirements, the following plan is recommended: The practicing veterinarian obtains from the state laboratory the mallein preparation, eye-droppers, test tubes, and needles for the collection of blood. On a special report blank the number, name, and position of the horses in the stable are noted. Then blood is collected (carefully considering the precautions mentioned in a special circular letter), marked in correspondence with the numbers on the report, and immediately forwarded to the laboratory. Simultaneously, a conjunctival test is made by dropping into the conjunctival sac (on the mucous membrane of the lower lid) two drops of a 5 per cent (1 per cent) solution of "mallein siccum." The solution must be made shortly before use. About 10-24 hours afterward, two examinations of the instilled eye are to be made. The degree of reaction is best marked on the report as follows:

Absence of reaction.....	N
A slimy discharge.....	D
A purulent discharge.....	P <sub>1</sub>
A purulent discharge and swelling of the eyelids.....	P <sub>2</sub>
An abundant purulent discharge with photophobia, lacrimation, etc.....	P <sub>3</sub> -P <sub>4</sub>

In cases where a doubtful reaction is obtained, a retest on the same eye with the same amount of mallein is made after the reading (20-24 hours after the first application). After 8-20 hours the eye is examined again and the result noted. All reacting animals are to be carefully isolated. In considering the result of the complement fixation and agglutination tests, the animals affected with glanders are proposed for destruction and if possible disposed of.

Fourteen days after the isolation, or better, the destruction, of the glandered horses, a third retest of the remaining animals, including, perhaps, the doubtful reactors, with ophthalmic mallein



and the serum tests is made. Should further cases of glanders be detected by the third, a *third repetition of the serum test alone* should be ordered. The subcutaneous mallein method should be omitted, or in case it has been applied, proper information should be sent to the one conducting the tests.

This plan for the diagnosing of glanders has been used with success during the last few months in the state of Pennsylvania.

## FUSIFORM BACILLI ASSOCIATED WITH VARIOUS PATHOLOGICAL PROCESSES.\*

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The occurrence of fusiform bacilli in connection with ulceromembranous anginas, ulcerative stomatitis, and noma is well known. The impression is obtained from the literature that these organisms are rarely found in morbid processes in other parts of the body.

In 1909, Ghon and Mucha<sup>1</sup> reported two cases of brain abscesses in which they found organisms of this type as the only demonstrable causes. Attempts at cultivation were unsuccessful. These abscesses were a part of a general pyemic process. In one case the primary infection was believed to be in the appendix; in the other case in a bronchiectatic abscess. In 1910, Kaspar and Kern<sup>2</sup> reported two more cases of pyemia in which fusiform bacilli were found and in one of these cases the bacilli were isolated in pure culture. In these cases the generalized infection followed appendicitis in one and a lung abscess in the other. In 1911, Peters<sup>3</sup> reported the finding of fusiform bacilli in smears in a case of lung abscess, in a case of fetid bronchitis, and in a case of hand infection. From a second case of hand infection, the bacilli were isolated in pure culture. In both hand infections, the wounds were caused by the teeth of other persons.

In 1911, Heyde,<sup>4</sup> in an examination of 102 cases of appendicitis, found fusiform bacilli in eight cases. In 1912, Rosenow and Tunnicliff<sup>5</sup> reported a case of pyemia in which pure cultures of fusiform bacilli were isolated from various lesions in the body. This case followed appendicitis with abscess formation.

It will be seen that the primary infections in all of these cases,

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<sup>1</sup> *Centralbl. f. Bakt.*, I, Orig., 1909, 49, p. 493.

<sup>2</sup> *Ibid.*, 1910, 55, p. 97.

<sup>3</sup> *Jour. Infect. Dis.*, 1911, 8, p. 455.

<sup>4</sup> *Ztschr. f. klin. Chir.*, 1911, 76, p. 1.

<sup>5</sup> *Jour. Infect. Dis.*, 1912, 10, p. 1.

excepting the infection of the hands from teeth, were either in the appendix or in the lung.

The following seven cases were examined postmortem by Dr. E. R. LeCount, to whom I am indebted for the opportunity to make the bacteriological examinations.

*Case 1.*—A man, 35 years old, was perfectly well up to five days before death. At that time he was taken with a severe headache which persisted. He vomited several times and had several chills. Was in the hospital about 24 hours, during which time the temperature ranged about  $101^{\circ}$ , pulse 80, leukocytes 25,000.

The anatomical diagnosis: Purulent and putrid meningitis, thrombophlebitis of left lateral sinus with necrosis of the temporal bone; chronic otitis media of left ear, acute follicular tonsillitis, hydropericardium, fibrous adhesive pericholecystitis, healed myocarditis.

The following is a description of the cranial cavity: The petrous bone on the left side contains a clot with a grayish-green color. There is necrotic tissue and pus in the middle ear. The right ear is unaltered. On removing the falx cerebri, there is a thick pus which flows out from the posterior part of the inferior longitudinal sinus. This is considerable in amount and foul-smelling. On the under surface of the cerebellum is a gray-green exudate which entirely obliterates the cerebellum. The posterior folds of the cerebrum are furrowed from the tentorium and pressure that existed under the tentorium and in the posterior part of the skull. The exudate is not marked about the circle of Willis but is apparent at the junction of the pons and medulla and at the vertex of the cerebrum. The convolutions are flattened. The sulci are not rounded but form right angles. The weight of the brain was 1,460 gms.

Bacteriological examinations: In stained smears of the purulent cerebrospinal fluid, many small gram-positive cocci in short chains and pairs were found. Slender gram-negative bacilli of lengths varying from a few microns to long, threadlike organisms were the only other organisms present.

Cultures were made on horse serum agar slants, made anaerobic by Wright's method, and in serum broth in an atmosphere of hydrogen. In the cultures on serum agar, the streptococcus alone

grew. It was a small gram-positive organism, the colonies of which resembled the streptococcus viridans. It grew more readily anaerobically than aerobically, and formed almost no green on blood agar. In the hydrogen serum broth cultures, bacilli resembling those of the smears were found. The long, threadlike forms predominated. From this serum broth culture, plates were made and incubated in jars from which the air was exhausted and the remaining oxygen removed as far as possible with pyrogallic acid and sodium hydrate. On these plates, several colonies of the bacilli developed in four days. These colonies were about two millimeters in diameter, flat, sharp in outline, and of a pearly translucent appearance. The bacilli in these colonies did not form such long filaments as in the broth cultures, were vacuolated, and in some instances swollen and irregular in size and shape. Attempts at subculture from these colonies failed. Various attempts were made to produce lesions in animals with the mixed cultures. Guinea-pigs were inoculated intracardially and the meninges and brain at the same time traumatized by perforating the skull with a stout needle. Rabbits were injected subcutaneously into the liver. A monkey was injected subcutaneously. In no instance was any pathogenic power for animals demonstrated.

*Case 2.*—This was a case in which no history was obtainable.

The following is the anatomical diagnosis: Fibrous adhesions between the left lung and chest wall, bronchiectasis of left lower lobe of lung, abscess in left occipital lobe of brain, fibrino-purulent basilar meningitis, hyperplasia of the spleen, cloudy swelling of liver, pancreas, and spleen, chronic interstitial nephritis, slight fibrous myocarditis with sclerosis of coronary arteries, marked emphysema of right lung and left upper lobe, passive hyperemia of liver, patent foramen ovale, fibrous epicardial patches, chronic mitral endocarditis.

The smears from the meningeal pus contained two kinds of organisms, a gram-positive lanceolate diplococcus and many long, threadlike organisms which did not retain the gram stain. Cultures were made on blood agar slants kept anaerobic by Wright's method and colonies of both the diplococcus and the gram-negative bacilli isolated. The diplococcus was identified as the pneumo-

coccus. The bacillus was found to contain a few coccus forms and dilutions were made in broth and the surface of blood agar slants seeded with these dilutions. In this way the bacillus, which formed long filaments on the surface of the blood agar slants, was isolated in pure culture. It grew only on serum ascites on blood media. The blood was not affected. It formed semi-translucent colonies easily removed from the surface of the agar. These colonies were hard to break up in liquids. Ascites gelatin was not liquefied. The growth appeared as a light opacity along the stab with no tendency to diffuse through the gelatin.

The organisms from the surface of slants were mostly slender spindle-shaped rods with many slightly longer curved forms and here and there long threads. Many beaded forms were seen.

The organism in pure culture was non-pathogenic for guinea-pigs. A large quantity of a mixed culture injected in a dog intravenously caused death apparently from intoxication from the pneumococcus present.

*Case 3.*—A man, 48 years of age, was admitted to the hospital complaining of severe headache. He had vomited a number of times, had been severely sick for two weeks, and had had a chronic bronchitis. The physical examination was negative except for deafness and bubbling rales over both lungs. The patient had two generalized convulsions of a clonic type and died a few hours after admission to the hospital.

The anatomical diagnosis at autopsy was: Cerebellar abscess, diffuse purulent leptomeningitis, sclerosis of the coronary arteries, fibrous myocarditis, bilateral catarrhal bronchitis, fibrous miliary tuberculosis of both pleurae, emphysema of both lungs, chronic catarrhal gastritis.

A description of the brain follows: On the middle of the under surface of the right lobe of the cerebellum there is an area of softening as large as a silver dollar. There is a foul-smelling, grayish-green pus exuding from this region. The convolutions of the brain are flattened and there is a slight amount of a grayish exudate in the meshes of the pia arachnoid. All of the minute vessels of the pia are engorged with blood. The fluid in the lateral ventricles is blood-stained. Numerous sections through all por-

tions of the cerebrum fail to reveal other gross lesions. Examinations of the sinuses, jugular veins, and middle ears and Eustachian tubes fail to reveal any gross changes. There is a thickening of the Eustachian tube on the right side. On a section of the cerebellum there is revealed an abscess cavity ( $4 \times 2$  cm.) occupying most of the right cerebellar lobe. It is filled with grayish-green, foul-smelling pus and surrounded by a grayish infiltration.

**Bacteriological examination:** In smears from the meningeal exudate two types of organisms. Small cocci, weakly gram-positive or gram-negative, in some instances were present in moderate numbers and great numbers of slender gram-negative bacilli for the most part about the length of the diameter of a pus cell or less. Many slightly longer forms were found. In many places large groups of these bacilli, as in colonies, were found. In the cultures, the streptococcus viridans and fusiform bacilli were isolated in mixed culture.

The fusiform bacilli grew well on blood agar slants but colonies free from cocci were not obtained. Mixed cultures injected intracardially in guinea-pigs in which the brain had been traumatized produced no lesions. The bacilli in the mixed cultures resembled in morphology those of cases 1 and 2.

**Case 4.**—A man, 43 years of age, fell and fractured the eighth and ninth ribs on the left side. Eleven months later he developed delirium tremens and bronchopneumonia. From that time until his death, three weeks later, he coughed up large quantities of mucopurulent material.

**The anatomical diagnosis:** Healing fractures of the eighth and ninth ribs, subpleural traumatic hemorrhages about the broken ribs, subparietal gangrene of left lung, pneumonia of both lungs, purulent and putrid serofibrinous pleuritis, compression atelectasis of the left lower lobe, emaciation, acute tracheobronchial lymphadenitis, diverticulum of the esophagus.

Smears from the pus show various gram-negative and -positive bacilli and cocci. There are many gram-negative slender pointed bacilli of all lengths up to threadlike filaments. From the cultures were isolated the staphylococcus albus and aureus, a pseudodiphtheria bacillus, a Friedlander bacillus, and a small gram-negative

bacillus similar to the colon bacillus. This bacillus grew only upon serum media anaerobically. It formed in four to five days an abundance of coal-black pigment.

In addition to these organisms, a fusiform bacillus was isolated in pure culture. The organism did not differ from the others described and the growth from six blood agar slants produced no lesions when injected into the testicle of a rabbit. Associated with the fusiform bacilli in the first subcultures was a streptococcus which occurred in short chains and pairs. It retained the Gram stain, grew better anaerobically than aerobically, did not affect blood and milk, did not ferment lactose, dextrose, mannite, or inulin.

*Case 5.*—The patient was a middle-aged man who died a few hours after admission to the hospital.

The anatomical diagnosis was as follows: Resolving pneumonia of the right upper lobe of the lung, unresolved pneumonia of the right lower lobe with multiple abscesses and gangrene, edema and hyperplasia of the tracheobronchial lymph glands, right-sided serofibrinous pleuritis, hemothorax from erosions of the lung produced by gangrene, partial compression atelectasis of the right lung, multiple miliary gummata of the capsule of the liver, hyperplasia of the lower esophageal lymph glands, cerebral softening, healing and healed luetic caries of the skull bones, marked sclerosis and calcification of the coronary arteries, slight fibrous myocarditis.

Bacteriological examination: The gram preparation of the pus itself showed large numbers of gram-negative fusiform bacilli of various lengths, many slightly wavy gram-negative spirilla, and some gram-positive streptococci. In pure cultures there were isolated the following organisms: *Streptococcus pyogenes*, a gram-negative slender bacillus with a marked tendency to pleomorphism growing only on blood media. It forms on blood agar slants growths in 24 hours which are slightly opaque and in four to six days become pigmented until jet black. *Fusiform bacilli*. This strain of fusiform bacilli did not differ from the ones from the other cases in morphology but grew on ordinary media to some extent. It did not affect milk, did not ferment lactose, glucose, inulin, or mannite. In lactose media, it tended to form longer filaments than on blood, was thicker, more vacuolated, and granular.

It was non-pathogenic for dogs when injected into the vein of a dog in large numbers. The spiral forms were not obtained in culture unless they are to be regarded as by Tunncliffe<sup>2</sup> and others as forms of fusiform bacilli. Some of the forms on lactose agar resembling the wavy forms found in the smear are suggestive of this view.

*Case 6.*—The patient was a man who died a few hours after entering the hospital. A huge foul-smelling empyema was found. The bacteriological examination showed the staphylococcus pyogenes albus, and fusiform bacilli. The bacilli did not differ from those described. The pathogenicity was not tested.

*Case 7.*—The patient died of a peritonitis following an abortion.

The following anatomical diagnosis was made: Corpus luteum of left ovary, parturient uterus, acute endometritis, erosion of the cervix, serofibrino-purulent peritonitis, marked cloudy swelling and fatty changes in the kidneys, cloudy swelling and fatty infiltration of the myocardium, cloudy swelling of the liver, petechial hemorrhages in the skin of the arms and right thigh, hyperplasia of the spleen, hyperplasia of the mesenteric lymph glands, recent laparotomy wound, old healed and incapsulated tuberculosis of the right apex, left fibrous pleuritis, anthracosis of the lung.

Bacteriological examination: The smears from the peritoneal exudate showed many gram-negative spindle-shaped bacilli, gram-positive bacilli, and streptococci. The following organisms were isolated in pure culture: *B. fusiformis*, *B. welchii*, *Streptococcus pyogenes*, a slender gram-negative bacillus which corresponded to the pigment-forming bacillus in case 4.

#### SUMMARY.

Fusiform bacilli were found in three cases of meningitis. In one of these cases, the infection probably arose from the middle ear which is in communication with the nasopharynx in which fusiform bacilli are commonly found. In the other two cases the infection probably followed chronic bronchitis, as in the cases reported by Ghon and Mucha and by Kaspar and Kern. Three cases of lung infection associated with fusiform bacilli are described, the first a bronchiectasis, the second a gangrene, and the third an empyema.

<sup>2</sup> *Jour. Infect Dis.*, 1906, 3, p. 148.



Fusiform bacilli were found in a case of peritonitis following an endometritis. The importance of the fusiform bacilli in causing the pathological processes with which they were associated, must remain an open question. The negative results of all attempts to demonstrate pathogenic powers for animals suggest that the organism may have been present only as a secondary invader, as other organisms were found in all the cases.

## FUSIFORM BACILLI. ISOLATION AND CULTIVATION.\*†

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Although an extensive literature exists on the fusiform bacillus, there are recorded no simple methods for the isolation and cultivation of these bacilli. The following methods were worked out in our endeavor to isolate a large number of strains for a comparative study.

The isolation from shake and surface cultures was tried. These methods were found unsatisfactory. The possibility of success depends on an abundance of bacilli in the original material or the examination of smears from colonies till one might chance on a fusiform colony. The use of surface growths was not seriously considered, as we were endeavoring to dispense with the use of pyrogallic acid. Shake cultures in capillary tubes were then made so that the character of the colonies could be studied under the microscope. This was also found unsatisfactory. The cultivation in agar contained between two layers of glass was then tried. For this purpose the two halves of the petri dish were sterilized so that the bottom was placed in the inverted cover. The inoculated ascitic agar was poured into the cover and the bottom of the dish was laid on top of the agar while it was still fluid. A very few threaded colonies were noted and fishing from these gave fusiform bacilli. This gave us the basis for future work.

The original material is suspended in about 5 to 7 c.c. of ascitic fluid or horse serum, and successive dilutions made in a series of tubes containing the same amount. In making the dilutions a pipette is used carrying over about 0.1 to 0.2 c.c. to make the dilutions gradual. About 15 c.c. of fluid agar is then poured into the tubes and the mixture poured in the manner already described (Fig. 1). During incubation the plates should be covered with paper to limit air contamination of the rim of exposed agar or the

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† This study had its inception in the interest of our associate, Dr. Matthias Nicoll, Jr., in the clinical conditions in which this organism is found. We are indebted to him for our material.

rim may be paraffined, which would also lessen the drying-out of the medium.

After incubation for 48 to 72 hours the upper layer of the dish is wrenched loose and the typical colonies searched for. These are characterized by threadlike outgrowths from one or both sides



FIG. 1.

of the colony. The general characteristics are shown in Figs. 2 and 3. This type of colony develops only in the agar, not between the agar and the glass. More agar is employed than in the usual plate; otherwise a good seal between the two parts of the petri dish is not obtained. For this reason, in fishing, the needle must pierce the agar nearly vertically or the small colony is missed. One way is to bend the end of the wire so that it pierces the agar vertically, and with a circular motion break up the colony. Other bacteria give a similar colony but they are not common in the material containing fusiform bacilli. In two instances we isolated from similar colonies a motile anaerobic strepto-bacillus.

For cultivation and preservation of stock cultures we have found the most satisfactory medium to be a semisolid mixture of gelatin and agar. The constituents are as follows:

Agar .....	10 gms.	} 2 parts.
Gelatin .....	80 gms.	
Broth... 2 per cent peptone, no salt, 1,000 c.c.		
Horse serum or ascitic fluid. ....	1 part.	
Slightly alkaline to litmus is a satisfactory reaction.		

Inoculation is made by stab. The advantages over agar are that the puncture closes better after inoculation, the medium does not split up on drying, and subinoculation is easy, due to the softness of the medium. Aerobic contamination is promptly noted by growth in the upper layer of the medium and a motile contaminant, likewise, would be evidenced by diffusion of the growth.

Although the various strains differ in their ability to grow on simple media, the only sure method of cultivating all strains is in the presence of serum. Horse serum is better than ascitic fluid,

possibly, because of the variations in the constitution of the latter. Freshly mixed media give much better results, probably because boiling the agar-gelatin medium drives off the contained oxygen.

As to the method of isolation, the ease with which strains may be isolated is evident from our results. Counting all the cases attempted, including those tried while perfecting our methods, we have isolated 18 strains in 42 trials. In our last seven cases we isolated a fusiform seven times.

None of the observations given have any priority. In a subsequent examination of the literature we found that Ellermann gives a picture of a similar colony and several authors speak of the



FIG. 2.—Colonies, 22 hours old.

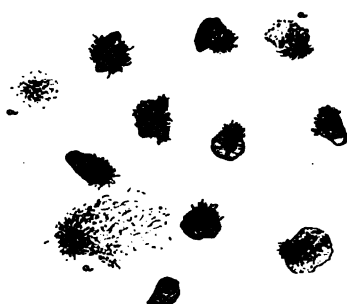


FIG. 3.—Colonies, 48 hours old. a, Surface or sub-surface colonies.

extensions from the colony, observations apparently made from pure cultures. No practical application of this fact was possible with the methods in use, should numerous colonies of other organisms develop. A priori we expected to find something distinctive in the colony, making this deduction from the morphology of the bacillus. The technic is given purely as a simple method requiring no apparatus and dispensing with cumbersome means of obtaining anaerobic conditions. As such it is the first simple practical method for isolation of these bacilli.

A study of the strains already isolated and the isolation of further strains for study as to their differentiation and classification, pathogenicity, relation of the fusiform bacillus to the associated spirochetes, and other biological characters is being made. The technical methods are given now in the hope that they will be of value to others interested in the subject.

## TWO METHODS FOR OBTAINING A VIRUS OF RABIES, FREED FROM THE CELLS OF THE HOST AND FROM CONTAMINATING ORGANISMS, AND THE APPLI- CATION OF THESE METHODS TO OTHER FILTER- ABLE VIRUSES OR GLYCERIN-EXTRACTS.\*†

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We have used two methods for obtaining a virus of rabies separated from the tissue cells of the host and freed from contaminating organisms. These methods we believe to be of value for other viruses, as for example, those of vaccinia, variola, epidemic-poliomyelitis, etc., and also of value in certain glycerin preparations or extracts such as tuberculin.

The first method is by glycerin-extraction, the second by aspiration.

### GLYCERIN-EXTRACTION.

Glycerin has long been used for destroying contaminating organisms and preserving the virus of rabies and vaccinia, etc., and of epidemic-poliomyelitis, more recently by Landsteiner, Levaditi, and Pastia.<sup>1</sup>

Glycerin is also of great value in certain extracts of bacteria and other preparations as in tuberculin. With tuberculin, several methods have been used for ridding it of glycerin, and thus allowing of a dry tuberculin: one is precipitation by alcohol and ether, which is costly; another is dialysis through parchment membrane. This dialysis is a comparatively slow process, difficult to perform under sterile conditions, and considerable of the tuberculin is lost.

No method, so far as we know, has been adopted for removing the glycerin from the viruses, after it has accomplished its

\* Received for publication February 10, 1913.

† Read at the meeting of the American Association of Pathologists and Bacteriologists in Philadelphia, April, 1912.

<sup>1</sup> *Ann. de l'Inst. Pasteur*, 1911, 25, p. 805.

purpose of preservation or destruction of contaminating organisms. Certain experiments on the nature of these viruses are complicated or rendered impossible by the presence of glycerin.

One of us<sup>1</sup> found that the virus of rabies could be extracted with glycerin from the submaxillary glands of rabid dogs. The glands were taken from the head under aseptic precautions. After removal of the fibrous sheath, they were immersed in sterile neutral glycerin and allowed to stand in the icebox for 6 to 12 days. The clear supernatant glycerin, slightly colored by hemoglobin, was then pipetted off. This was usually found to be sterile, and microscopical examination of this fluid showed practically nothing demonstrable in the way of formed cellular elements. This glycerin-extract retained its virulence, when kept in the icebox, for a very long time, in one experiment 191 days.

No further work was done on this extract, due to the limitation of experimentation with a glycerin virus.

By our present method—dialysis through collodion sacs—we are able to get rid of the glycerin and have our virus uncontaminated in any fluid we select.

We do not say that the dialysis of glycerin is a new method, as it is a well known fact, but its application, as far as we can ascertain, has been through parchment or parchment paper. The objections to this we have already mentioned in connection with tuberculin.

By the use of the collodion sacs, all the operations are carried on under aseptic conditions. The usual contaminating organisms do not pass through a collodion sac. Therefore if the sac is sterile and the glycerin extract put in the sac is pure, the fluid in which it is placed for dialysis need not be sterile.

Another advantage of the collodion sacs is that dialysis of glycerin takes place very rapidly in physiological salt solution, Ringer's solution, or distilled water. For example, the glycerin, from 4-5 c.c. of the glycerin virus of rabies, could be removed in one to two hours' dialysis in running salt solution.

A third advantage of the collodion sac is that it may also be used to concentrate the virus. After dialysis the virus is diluted,

<sup>1</sup> Poor, *Proc. of the New York Path. Soc.*, 1906, 6, p. 85.

the dilution varying with the amount of water the glycerin has previously extracted from the tissues.

In microscopical studies or other work, a concentration may be desired. Evaporation may not be practicable, and as most of the filterable viruses do not filter through a collodion sac, by using the sac as a filter we are able to get rid of the diluting fluid. Although some of the virus may adhere to the side of the sac, a certain concentration must result.

It is true, also, that, although the protein in solution in the sac will gradually pass through as the filtration continues, due to the "coating" of the sac, the protein content of the fluid, remaining within the sac, when the concentration has reached the desired degree, will be increased, as shown by one of us.<sup>1</sup> In interpreting some of the results of concentration, this fact must be borne in mind.

The technic used for making the sacs was that described by Novy<sup>2</sup> and modified by Gorsline.<sup>3</sup>

The sacs were made over a tube which had a small opening in the bottom. This tube was turned several times in a dilute collodion. This collodion was then stripped off by forcing water through the tube. A glass tube was inserted into the neck of the sac, and the junction strengthened by a ligature or a coating of collodion. The sac was filled with distilled water, autoclaved at 105 degrees for 15 minutes, allowed to cool, then emptied, immersed in sterile water, and subjected to pressure (about three inches of mercury). If there was no evidence of leakage, the sac was used. The sacs were without flaws or air bubbles. For dialysis, they were fairly thick; for filtration, they were very thin.

#### THE ASPIRATION METHOD.

This method was also used by us in our studies on the rabies virus of the submaxillary glands. The glands, after being cut, were placed in distilled water and subjected to a vacuum of 29 inches of Hg for an hour. The pieces of the glands were then pressed in an ordinary meat press and the expressed fluid centrifuged.

<sup>1</sup> Steinhardt, *Jour. Infect. Dis.*, 1910, 7, p. 675.

<sup>2</sup> *Bact. Lab. Handbook*, 1898.

<sup>3</sup> *Contrib. Med. Res.*, Ann Arbor, Mich., 1903, p. 390.

The supernatant fluid was pipetted off and added to the water in which the glands were aspirated. It may be noted that the aspiration fluid alone was found to contain the virus, but more was present on the addition of the pressed-out juice. By this method a suspension of the virus was obtained in distilled water, which was stronger than the glycerin virus, but which was not sterile and which contained fragments of glands, cells, blood-tissue elements, etc. These were removed by passing it through a Berkefeld filter, the filtrate containing the virus, freed from tissue cells and contaminating bacteria.

#### CONCLUSIONS.

Our two new methods are: first, the application of collodion sacs for the dialysis of glycerin; second, the use of aspiration for obtaining the virus of rabies from the submaxillary glands of rabid dogs. This aspiration extract is virulent when filtered through a Berkefeld filter.

These methods have enabled us to obtain a virus of rabies freed from the cells of the host and other contaminating organisms and to suspend this virus in any desired fluid.

The results of our studies on rabies will be published shortly. We have also done some comparative work with vaccinia, and are beginning similar studies with epidemic-poliomyelitis, the virus of which, as frequently noted, has a strong likeness to that of rabies.

For obtaining a dry tuberculin from the glycerin preparation, we believe the use of the collodion sacs for dialysis to be superior to other methods.



## STUDIES ON THE VIRUS OF HOG CHOLERA.\*†

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### INTRODUCTION.

The terms "filterable virus" and "ultramicroscopic organisms" are frequently used in the descriptions of some 30 important infectious diseases of man and animals. These terms constitute a convenient form of expression, as in certain cases it would otherwise be necessary to state "specific cause unknown."

Among the important diseases which fall into this class, as enumerated and briefly described by Wolbach,<sup>1</sup> are yellow fever, molluscum contagiosum, dengue fever, verruca vulgaris, trachoma, sand-fly or three-day fever, acute anterior poliomyelitis, measles, typhus fever, and scarlet fever, which affect man only; rabies, foot and mouth disease, variola, and vaccinia affect both man and animals; whereas hog cholera, fowl pest, cattle plague, sheep pox, African horse sickness, swamp fever of horses, and Rous's chicken sarcoma are among those affecting animals only. Canine distemper, which Wolbach mentions as being produced by a filterable virus, has been demonstrated by Ferry<sup>2</sup> to be due to a bacillus termed by him *B. bronchisepticus*.

Since the recognition of the filterability of hog cholera virus by De Schweinitz and Dorset, 1903-1905, very little progress has been made in elucidating the nature of the specific etiological factor involved.

Dinwiddie,<sup>3</sup> from a series of carefully planned centrifugation experiments, concludes that the infectious agent involved in hog cholera is more or less closely associated with the red blood corpuscles. Aside from the report of Dinwiddie, the knowledge

\* Received for publication February 15, 1913.

† Read before the Society of American Bacteriologists, January 2, 1913.

<sup>1</sup> *Boston Med. and Surg. Jour.*, 1912, 167, p. 419.

<sup>2</sup> *Jour. Infect. Dis.*, 1911, 8, p. 399.

<sup>3</sup> *Bull.*, Arkansas Exp. Sta., 1912, No. 111.

accumulated in regard to this subject is relatively meager and scattered.

The filterability of a given virus does not preclude the possibility of the presence in that virus of relatively large microorganisms at certain periods, according to the stage of development in their life cycle. Borrel<sup>1</sup> demonstrated a protozoan, *Micromonas mesnili*, which, during certain stages of its development, would pass through the Berkefeld and Chamberland filters.

Loeffler also has shown that a flagellate belonging to the genus Bodo would pass through Berkefeld filters, which were capable of preventing the passage of *B. prodigiosus*, even after an hour's filtration. Thus, filterability cannot be taken as an indication of the size of a given organism, because the passage of organisms through filters depends also on their plasticity and their ability of accommodation to the pores of the filter. In this, the smaller forms of motile parasites differ from bacteria of relatively the same dimensions. It therefore seems possible that some of the filterable viruses causing disease may have a stage in the cycle of their development which is visible under the microscope.

Betegh<sup>2</sup> of Hungary has succeeded in retaining hog cholera virus by means of the Bechhold ultra-filter. His experiments consisted in the filtration of two different strains of virus and the injection of each of the filtrates into three susceptible pigs. The six pigs remained well and the conclusion was that the virus had remained in the ultra-filter.

In 1910,<sup>3</sup> a comparative histological study of the blood of normal hogs and cholera-infected animals was conducted. In this work, the blood of hogs suffering from cholera, in comparison with normal blood, was studied in a routine way on the hemocytometer and with the ordinary blood stains, but at the same time much care was exercised in attempting to find any differences which might indicate the probable nature of the causative factor. Aside from the data obtained relative to the ordinary blood examinations, nothing of note was observed in the specimens of hog cholera blood.

A few months ago a comparative study of normal hog blood and

<sup>1</sup> *Ann. de l'Inst. Pasteur*, 1903, 17, p. 81.

<sup>2</sup> *Ber. thierärztl. Wchnschr.*, 1912, 28, p. 969.

<sup>3</sup> King and Wilson, *Bull. Kansas Exp. Sta.*, 1910, No. 171.

blood from animals suffering from hog cholera was undertaken, in order to determine whether any differences could be detected by means of the dark field method of illumination. A preliminary report<sup>1</sup> of this work has appeared.

The fresh blood of normal and diseased hogs was collected aseptically in sterile sodium citrate solution and kept in the incubator until the examination was completed. The blood was obtained from the caudal artery after a small portion had been clipped off with a sterile instrument. In this way, bacterial contamination was reduced to the minimum.

In the blood of both normal hogs and animals suffering from hog cholera, as in the blood of any animal, many peculiar structures are observable. The blood of normal hogs thus examined on the dark field may show, in addition to the normal structures, a few granular bodies, sometimes a few bacterial cells from possible extraneous contamination and filaments. The latter may assume the form of "dumb bells," "chains," "droplets" or flexible filaments, which by an untrained observer might easily be mistaken for spirochetes. An excellent description of these bodies as well as others found in the blood of normal animals is given by Balfour.<sup>2</sup>

Betegh, in examining the hog lymph and serum respectively, from two animals dead from hog cholera, evidently has mistaken some of these filaments, described by Balfour, for bodies of some possible significance. In his very recent article on ultrafiltration experiments, Betegh<sup>3</sup> describes, in a rather confused way, his limited dark field findings as follows:

"Series I. January 16, 1912. Animal infected and died with hog cholera under natural conditions. Pathological changes: Typical stratification (button ulcers) in cecum and colon; edematous infiltration along the arteria coronaria cordis; infiltration in the right apex of lung; disseminated infiltrated areas in the right lung; parenchymatous degeneration of the kidneys; hemorrhagic inflammation of the lymph glands. Diagnosis: Hog cholera. Microscopic findings: Scattered bipolar and other (colon) bacilli in the organs. In the lung lymph beside these, small slightly stained bodies. In the dark field examination, actively motile, massed bodies from 0.3 to 0.5 microns in size, are visible, which are strongly light-refractive. Many appear to have a small apophysis. Very often dumb-bell like forms are seen, and further, spirochete-like microorganisms, which have at each end a light-refracting round knob. Cultural: On

<sup>1</sup> King and Baeslack, *Jour. Infect. Dis.*, 1913, 12, p. 39.

<sup>2</sup> *Fourth Report*, Wellcome Tropical Research Laboratories, 1911, p. 109.

<sup>3</sup> *Berl. thierärztl. Wchnschr.*, 1912, 28, p. 972.

alkaline agar these have grown at 37° after 24 hours, many coli, bipolar, and bacterial colonies similar to the *B. suispestifer*."

"Series II. April 16, 1912. Swine sickened and died under natural conditions. In colon a typical button ulcer of the size of a small coin. Several lentil-sized ulcers at the point of anastomosis of the small intestine into the colon. The mesenteric lymph glands swollen and hemorrhagically inflamed. The apex of both lungs infiltrated, atelectasis. The pleura was weak and covered with pseudo-membranes. Surface of incision of the lungs marbled; beside yellow necrotic groups, different stages of hepatization of partial pneumonia were visible. Diagnosis: Hog cholera. Microscopically with diluted carbol fuchsin solution, numerous, short bacteria, rounded off at both ends, occasionally typically bipolar colored, were visible. With the Giemsa staining, in part, the same form. Numerous 0.3 to 0.5 micron sized, ovoid or round forms were seen, which seemed to arise from a chromatin substance. In colored streak preparation from the button ulcers, intra- and extra-cellular typical spirochetes were seen. Similar bodies were abundantly visible with the dark field in the serum. They were actively motile. Between small dumb-bell shaped forms were seen also spirochete forms."

From the above description it is quite evident that Betegh based his observations on the fallacies to which Balfour has so aptly called attention. In the blood of different species of normal animals, filaments may be observed which at times closely simulate spirochetes. Most of these "pseudo-spirochetes," as seen in blood on the dark field, present a knobbed appearance at each pole. After one has become accustomed to the appearance of these filaments, one can readily distinguish the lack of motility, in spite of a flexuous distortion, which is assumed as the filaments are carried by a slight current in the fluid of the preparation. These filaments are also lacking in refractibility and are relatively slender in comparison to the spirochetes recognized in this work.

Bacilli are easily recognized in the dark field by the complete rigidity of the cells and, when flagella are present, by the characteristic tumbling motion.

Results from the dark field examination of specimens of blood from some 50 normal hogs indicate that, as a rule, the blood is relatively free from granules. Under ordinary conditions, and with few exceptions, the blood of normal hogs examined has been designated in our notes as "clean." There have been some few instances in which the blood of hogs unexposed to cholera, and in apparent normal condition, have shown the presence of numerous granules. The presence of these granules in normal blood, in the majority of cases, has been traced to ruptured leukocytes.

In the specimens of blood from all infected hogs, which have been observed by means of the dark field, a relatively large spirochete has been found. It averages from five to seven microns in length and one micron in width. The body of the organism is flexible and round at its ends. It presents no knobbed appearance

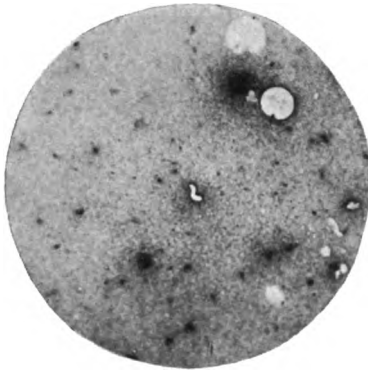


FIG. 1.—Microphotograph of spirochete in blood of Hog 504. India ink preparation.

at its poles. Actively motile, it revolves about its longitudinal axis. Its motility is undulating in character and its spirals are fixed. A few of these organisms have been observed dividing longitudinally. In one permanent microscopical mount, prepared by india ink fixation, one of these organisms apparently shows a polar flagellum. On the dark field this spirochete is readily distinguished from bacteria on account of its lack of rigidity

and its characteristic motility, and from "blood filaments" by its greater refractive properties and characteristic morphology.

This spirochete has not been found in large numbers, in any of the blood preparations. However, in nearly every specimen examined, more than one has been observed and in many cases five or six have been found with little difficulty. As a rule the organisms have been found to be more numerous at the height of the disease. The specimens of blood examined have been diluted in the proportion of about one to ten or fifteen with sterile sodium citrate solution, which factor should be considered in contemplating the number observed in a given positive specimen. Moreover, it is suggested that this organism, when observed as a spirochete form, constitutes only one stage of its development.

Certain types of granules appear to be characteristic of blood from cholera hogs. It usually contains many granules, some very fine yet more distinct than blood dust, some larger still, and some very distinct, highly refractive bodies. In many specimens of cholera blood were observed innumerable small granules, which

were much more definite and distinct in outline than blood dust, and easily differentiated from the whitish, partially refractive granules from ruptured leukocytes and from the more highly refractive and larger bodies composed of débris, bacteria, and filaments. In this work the presence of these granules appeared to be so characteristic of blood from cholera hogs, that it became an invaluable aid in finding the spirochetes. While these particular bodies may be disintegrated blood elements resulting from disease processes, yet it may properly be suggested that some of them may represent certain stages in the life cycle of the spirochete which has been observed.

The majority of the hogs used in these experiments were inoculated with virus which was diluted 1 to 4 with sterile physiologic salt solution and filtered through Berkefeld filters. In the filtrates only a few granules were visible when studied on the dark field.

In this connection it should be mentioned that the majority of the dark field examinations which are included in this work have been checked by each of us. In addition to the above means of controlling the results, specimens of blood were at times collected by an assistant who designated the specimens by symbols. As the daily examinations often included specimens from both normal and diseased animals, the results were put to a practical test and, in no instance, did the results from the dark field examinations deviate from the clinical conditions of the animals whose blood was examined. In some instances, as will be shown by the following detailed results, the presence of hog cholera infections was practically detected by the dark field examination before it was known that the animals showed any symptoms of the disease.

It is interesting to note that in 1894 Dr. Theobald Smith<sup>1</sup> submitted the following brief report on "Coarse and Fine Spirilla in the Intestine of a Hog":

"The recently published articles on fine spirilla in the excrement of cholera subjects prompt me to communicate an observation from animal pathology. Early in 1889 I found non-liquefying comma bacilli in small abscesses of the large intestine, in a hog which I have briefly described in this journal. In streak preparations of the same, stained with alkaline methylene blue, I found, besides large quantities of vibrios, also

<sup>1</sup> *Centralbl. f. Bakt.*, 1894, 16, p. 324.

a great many fine spirilla, of two to three wave-lengths. The wave-lengths of fixed form are about two microns. A preparation, now more than five years old, still shows the tiny spirilla very distinctly. At that time they never appeared again in cultures. Further investigations concerning the presence of this organism in other animals have not been made."

Betegh, in a lecture and demonstration given before the Veterinary Congress at Budapest in May, 1912, called attention to the spirochetes which were demonstrable in the ulcers of animals dead from hog cholera.

Little difficulty has been experienced in finding spirochetes in

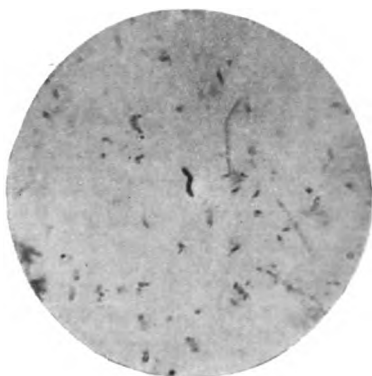


FIG. 2.—Microphotograph, spirochete stained by Giemsa method, from intestinal ulcer of Hog 556.

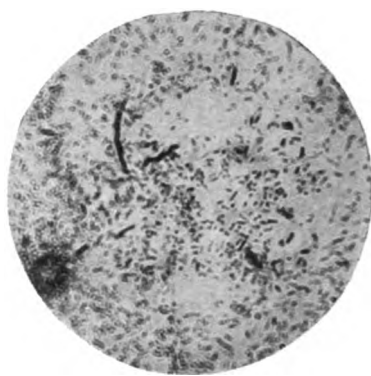


FIG. 3.—Microphotograph of spirochetes in impure culture, from cecal ulcer of Hog 551.

the intestinal ulcers of hogs dead from cholera. In the present work the ulcers, as found in the cecum of cholera-infected hogs, have been obtained as free from contamination as possible, washed with sterile water, and intermittently scraped with sterile instruments. Portions of the scrapings from the diseased submucosa, after the above treatment, have then been examined on the dark field and in stained preparations, preferably by the Giemsa method. While it thus appears possible to demonstrate spirochetes in hog cholera ulcers with uniformity, yet a large variety of bacterial species of course constitute the flora. With present methods of study therefore, the recognition of these spirochetes in the intestinal lesions cannot be taken as of great significance. Whether or not some of these spirochetes in the intestinal lesions bear any relation to those present in the blood of cholera hogs is, of course, an open question.

It is possible that the spirochetes found in the blood enter the lymph and blood stream at an early stage of the ulcer formation. This would offer a possible explanation of their comparatively small number as seen on any one blood mount.

The details of the experiments may be better understood by a study of the following temperature charts and laboratory notes. The temperature charts include the time of inoculation, death or release of each animal, and other necessary data including brief notations of clinical conditions and changes observed after death. The results of the dark field examinations are indicated by positive and negative signs placed above the temperature curves on the dates when the observations were made. In some instances the presence of granules or freedom of the blood from granules is indicated by abbreviations, gr. (granules) and c. (clean).

*B.A.I. strain of virus.*—The history of the strain of virus, designated in these notes as the "B.A.I. strain of virus," was supplied by Dr. Giltner of the Michigan Agricultural College as follows: "This is a representative of the original strain of Bureau of Animal Industry virus secured by Dr. Marshall at the conference at Ames in May, 1908. We have never kept any other strain of virus in the laboratory for any length of time. Practically all of our serum has been produced on the basis of this virus. It would be possible but very difficult to trace the passages through which this virus has gone since we first began to use it."

Beginning with Hog 446 the blood of practically all animals used in these experiments was carefully examined before inoculation in order to control the results.

Hog 446, inoculated with the B.A.I. strain of virus (Hog 444), manifested symptoms of the acute type of hog cholera after an incubation period of seven days. Three positive findings were made in the blood of this animal.

Results from the dark field examinations of the blood of Hogs 453 and 454 afforded most interesting data. Both of these animals recovered after a mild course of the disease following a relatively long period of incubation. As the symptoms in both animals disappeared, dark field examinations failed to reveal the presence of the spirochete and the numerous characteristic granules. These hogs were kept under observation during a period of one month following recovery and were ultimately released as "immune."

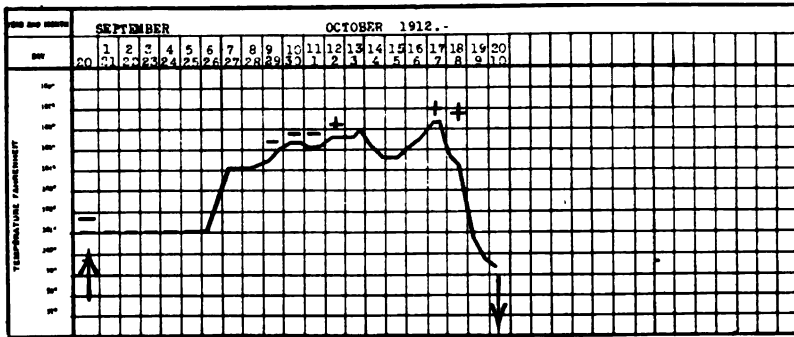
Hog 455 was kept in a separate room in the experimental stable from October 26 until November 10 for the purpose of serving as a control on the methods used in isolating the experimental lots of hogs. During a part of this period Hog 455 suffered from a parasitic skin affection, but dark field examinations demonstrated a "clean" blood, free from granules and the spirochete. On November 10 the animal was exposed to the disease and developed symptoms in six days. His blood then showed positive findings and autopsy revealed lesions of cholera.

The charts of Hogs 506 and 507, inoculated with the B.A.I. strain of virus, need no further explanation.

*Michigan (Demerick) strain of virus.*—On September 24 a farm at Roseville, Mich., was visited for the purpose of examining the herd of hogs. Several sick hogs of this herd, which had been isolated, manifested symptoms similar to those of hog cholera—malaise, anorexia, high fever, and diarrhea. A few purple areas were observed on the abdomen and ears.



## HOG No. 446. B.A.I. STRAIN OF VIRUS.



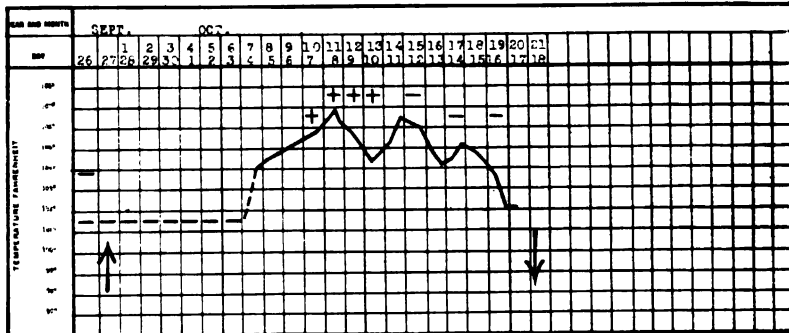
REMARKS: September 20. Animal inoculated with 3 c.c. from 444 (B.A.I.).

September 27. Symptoms appeared.

October 10. Animal moribund, bled and examined.

Skin on ventral surface of body purple in color; hemorrhagic areas in subcutaneous and muscular tissues. Lymphatic glands enlarged and hemorrhagic. Kidneys show a few petechia, ulcers in cecum.

## HOG No. 449. B.A.I. STRAIN OF VIRUS.



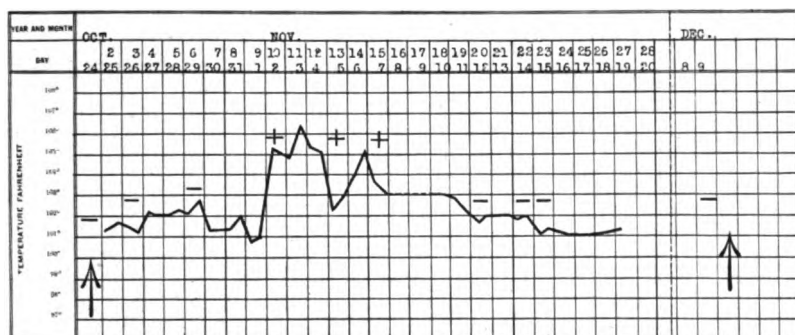
REMARKS: September 27. Inoculated with 4 c.c. B.A.I. virus.

October 5. Symptoms appeared.

October 18. Animal found dead.

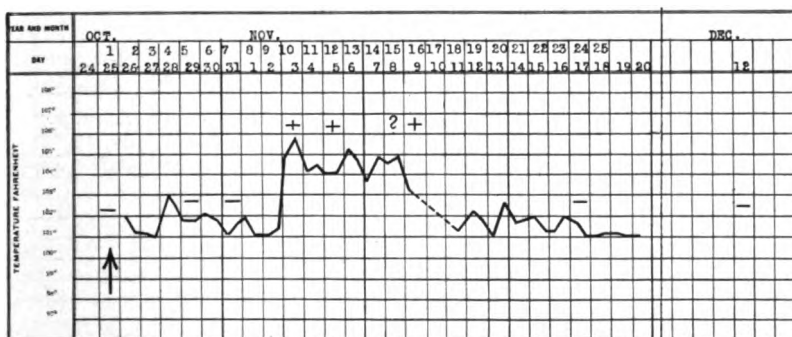
Purple hemorrhagic areas extending over greater portion of body externally and into subcutaneous tissue internally. Lymphatic glands enlarged and hemorrhagic. Ulcers in cecum. Kidneys petechiated. Areas of fatty degeneration in liver, right lung, upper lobe solidified.

## Hog No. 453. B.A.I. STRAIN OF VIRUS.



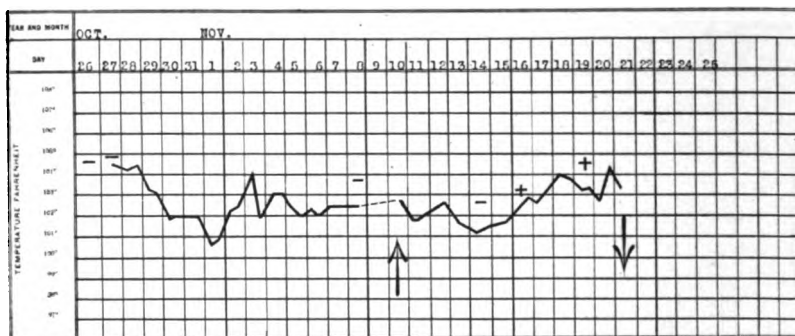
REMARKS: October 24. Inoculated with 4 c.c. B.A.I. (filtered) virus, filtered through Berkefeld.  
 November 2. Symptoms appeared.  
 November 5. Blood positive.  
 November 19. Animal recovered.  
 December 15. Animal released, immune.

## Hog No. 454. B.A.I. STRAIN OF VIRUS.



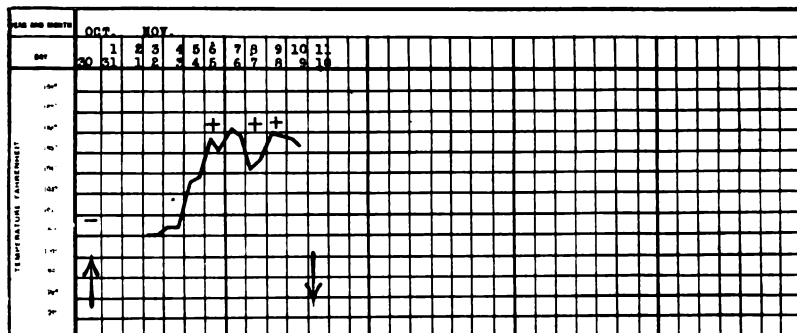
REMARKS: October 25. Inoculated with 4 c.c. B.A.I. virus (unfiltered).  
 November 2. Symptoms appeared.  
 November 19. Animal recovered.  
 December 15. Animal released, immune.

## HOG No. 455. B.A.I. OR MICHIGAN (DEMERICK) STRAIN OF VIRUS.



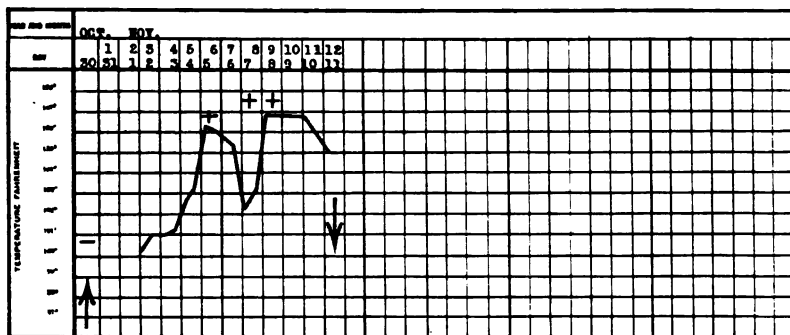
REMARKS: *Control.* October 28. Appearance of "rash" on abdomen and flanks. Animal eats well and shows no symptoms of cholera. Diagnosed as parasitic skin affection.  
 November 10. Recovering hogs (not dipped) 446, 447, 448 placed in inclosure with 455.  
 November 16. Animal sick.  
 November 21. Pig moribund, bled and examined.  
 Typical lesions in cecum, lymphatic glands, and lungs.

## HOG No. 506. B.A.I. STRAIN OF VIRUS.



REMARKS: October 30. Inoculated with 4 c.c. B.A.I. virus.  
 November 4. Symptoms appeared.  
 November 10. Animal found dead and examined.  
 Cecum, lymphatic glands, spleen, kidneys, and lungs show typical lesions of cholera.

## Hog No. 507. B.A.I. STRAIN OF VIRUS.



REMARKS: October 30. Inoculated with 4 c.c. B.A.I. virus.

November 4. Symptoms appeared.

November 11. Animal found dead and examined.

Lymphatic glands, cecum, lungs, kidneys, and spleen show typical lesions.

With the permission of the owner two of the animals were bled from the carotid artery and careful examinations made. Typical lesions of hog cholera were found in the large intestines, lymphatic glands, lungs, kidneys, and spleen, and a positive diagnosis of the disease in the acute form was made.

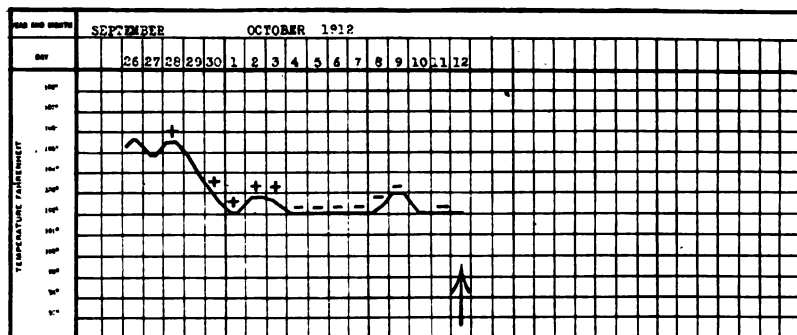
On September 26 two animals affected with hog cholera were brought to the laboratory. The blood of one of these hogs was examined immediately and the spirochetes were found with little difficulty. This animal was moribund, and was bled and examined on the same day. The other hog, No. 448, ultimately fully recovered from the disease. An examination of the temperature chart of Hog 448 will show the logical results obtained from the study of his blood on the dark field.

Three hogs, Nos. 450, 451, and 452, were inoculated with the Michigan (Demerick) strain of virus. The results of the dark field blood examinations appear in the charts below and were clearly confirmatory of previous findings.

*Michigan (Rochester I) strain of virus.*—On November 9, eight apparently normal pigs were received from Rochester, Mich. No particular notice was taken of these animals as the blood of other hogs suffering from cholera was at the time under close observation. The eight susceptible pigs were received in an isolated pen, at some distance from the experimental stable, and cared for by an attendant who never entered the experimental stable. This was the routine procedure when normal pigs were received.

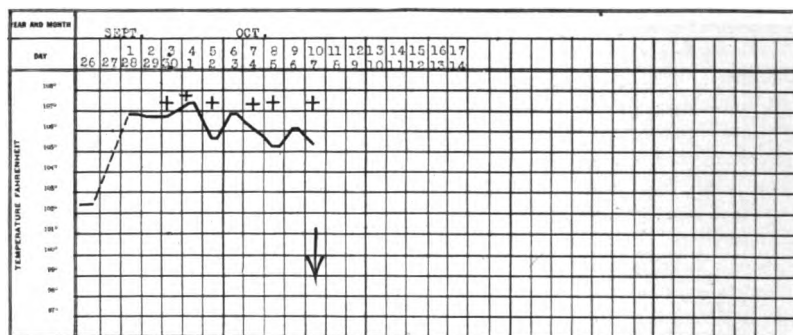
On the evening of November 12, Hog 503, one of the eight apparently normal pigs from Rochester, was removed to the experimental stable, inoculated with Michigan (Demerick) strain of virus and placed alone in a disinfected room. In this instance no dark field blood examination was made before inoculation, owing to the lack of time. It was planned that the examination of the blood, for the purpose of control, should be made the next morning following the inoculation. Likewise no temperature observation was made until the next morning. On November 13, the day following the inoculation of Hog 503, the dark field examination of the blood revealed the spirochete.

## HOG No. 448. MICHIGAN (DEMERICK) STRAIN OF VIRUS.



REMARKS: October 12. Animal fully recovered. Released as immune.

## HOG No. 450. MICHIGAN (DEMERICK) STRAIN OF VIRUS.



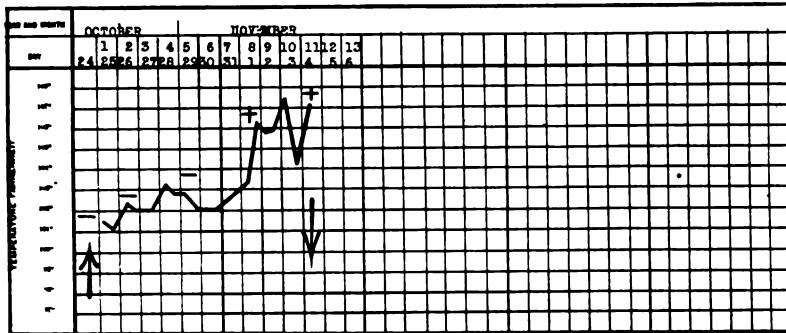
REMARKS: September 27. Animal inoculated with 4 c.c. Dimerick virus.

October 5. Symptoms appeared.

October 14. Animal found dead and examined.

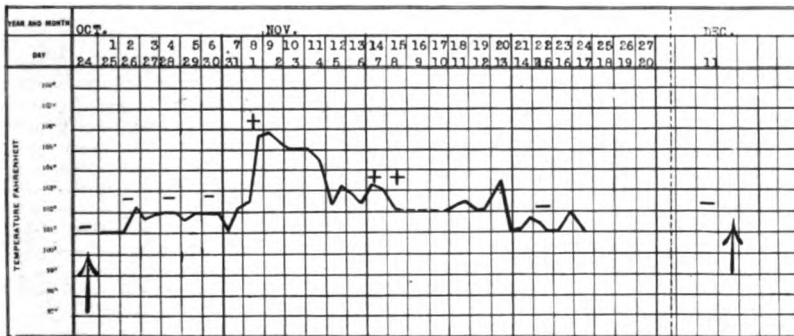
Large areas of congestion and hepatization in lower lobes of both lungs, kidneys contain a few petechiae. Spleen enlarged and gorged with blood. Mucosa of large intestine congested. Lymph glands enlarged and hemorrhagic.

## HOG No. 451. MICHIGAN (DEMERICK) STRAIN OF VIRUS.



REMARKS: October 24. Demerick virus (filtered through Berkefeld).  
 November 1. Symptoms developed.  
 November 4. Animal found dead and examined.  
 Lesions in lymphatic glands, spleen, lungs, and cecum typical of cholera.

## HOG No. 452. MICHIGAN (DEMERICK) STRAIN OF VIRUS.



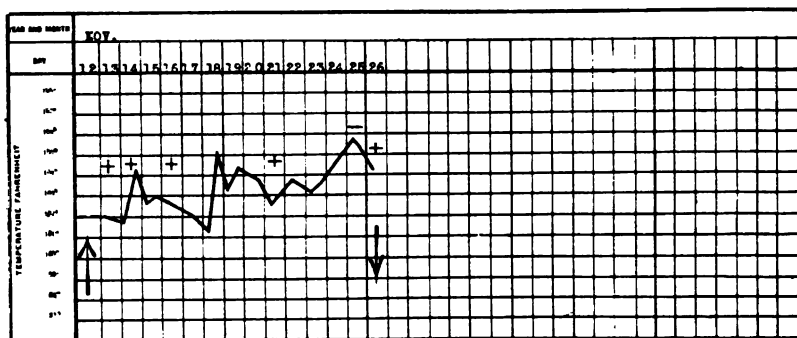
REMARKS: October 24. Inoculated with 5 c.c. of Michigan (Demerick) virus (unfiltered).  
 November 1. Symptoms appeared.  
 November 17. Animal recovered.  
 December 15. Animal released, immune.

The organism was again found on November 14. At this time the temperature of the animal was 104.2° F. and it showed symptoms of hog cholera. An inspection of the remaining hogs of this lot, which were still in the isolated pen used for receiving susceptible animals, showed that practically all were suffering from the disease. Among the apparent symptoms were diarrhea, anorexia, and listlessness.

Careful inquiry was made as to the source of this lot of pigs and it was found that they were purchased from a stock buyer, who had kept them for 10 days previous to receipt, in an inclosure which received all hogs purchased. Thus, it was clear that these pigs had been subjected to one of the most common methods of exposure to the disease, and that an incubation period of 10 days had elapsed before they were delivered at the laboratory.

In this instance a diagnosis of hog cholera was practically made by means of the dark field.

#### Hog 503. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



REMARKS: November 12. Inoculated with 4 c.c. virus from Hog 450 Michigan (Demerick) strain.  
 November 14. Found pig showing symptoms. One of Rochester hogs.  
 November 17. Typical symptoms.  
 November 20. Typical symptoms.  
 November 26. Animal moribund, bled and examined.  
 Lymphatic glands, lungs, kidneys, and heart muscle show typical lesions.

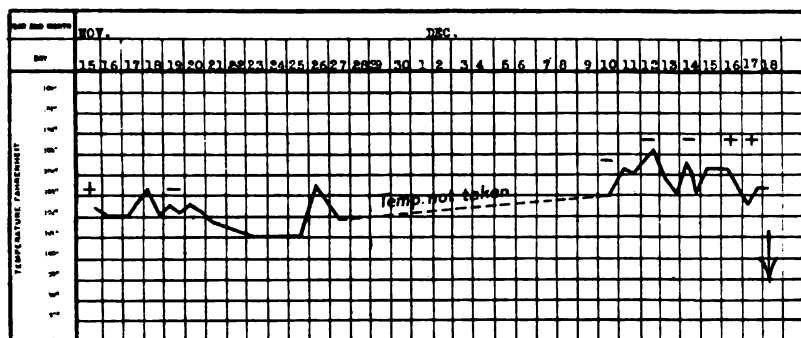
The autopsy findings in Hog 503, which was bled two weeks after being taken under observation, could not consistently be used to verify the diagnosis of cholera in this lot of hogs, because of the fact that this animal had received an inoculation of Michigan (Demerick) strain of virus.

Hog 504 was killed and examined on November 16, after two positive dark field examinations were made. The lesions were not pronounced because of the fact that sufficient time had not elapsed for typical lesions of cholera to develop.

The disease in this lot was of the subacute or chronic type as is shown by a study of the following charts of Hogs 505, 509, 510, 511, and 513. The clinical conditions and autopsy findings left no room for doubt as to the nature of the disease.

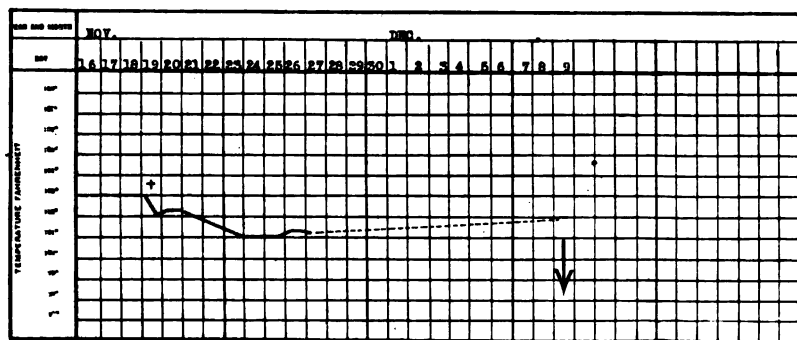
In further confirmation of the diagnosis of hog cholera in the above animals, when received at the laboratory, attention should be called to the chart of Hog 514. This animal remained in the isolated pen for susceptible hogs at the time the Rochester pigs

## HOG No. 505. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



REMARKS: November 15. One of Rochester pigs. Not inoculated.  
 November 20. Symptoms of chronic type.  
 December 10. Animal very weak and emaciated.  
 December 19. Hog died on bleeding table.  
 Lymphatic glands, heart muscle, lungs, kidneys, and cecum show typical lesions of cholera.

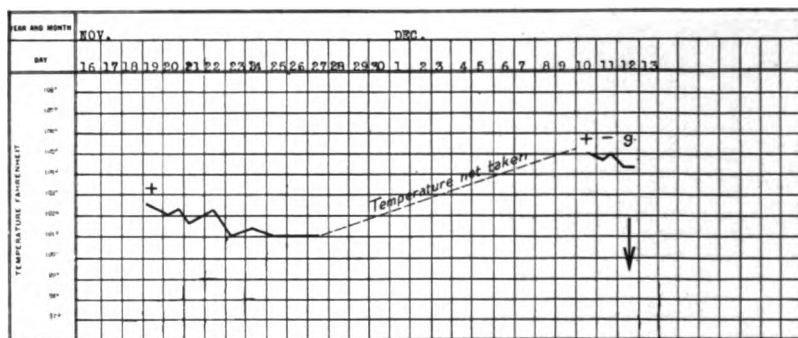
## HOG No. 509. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



REMARKS: November 16. Rochester pig. Not inoculated.  
 November 20. Symptoms of chronic type.  
 December 9. Animal found dead.  
 Lymphatic glands, lungs, spleen, heart muscle, and kidneys show typical lesions.

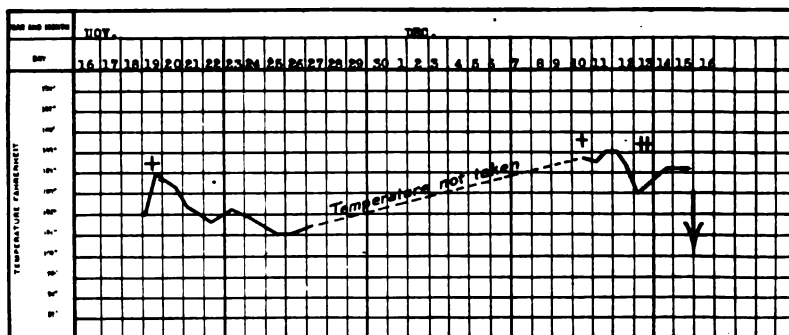


## Hog No. 510. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



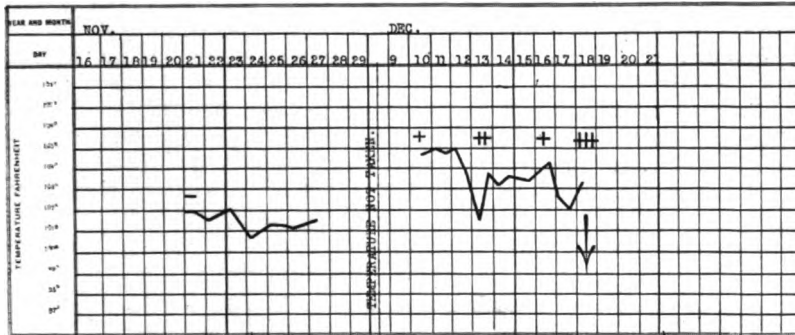
REMARKS: November 16. Rochester pig. Not inoculated.  
 November 20. Symptoms of subacute type.  
 December 10. Animal very weak and emaciated.  
 December 12. Animal moribund, bled and examined.  
 Spleen, lymphatic glands, and cecum show typical lesions of cholera.

## Hog No. 511. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



REMARKS: November 16. Rochester pig. Not inoculated.  
 November 20. No marked symptoms, looks fairly good.  
 December 1. Symptoms of subacute type.  
 December 10. Animal very weak and emaciated.  
 December 16. Found dead.  
 Typical lesions, lymphatic glands, kidneys, lungs, and cecum.

## HOG No. 513. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



REMARKS: Rochester pig, not inoculated.

**November 20. No marked symptoms, but not doing well.**

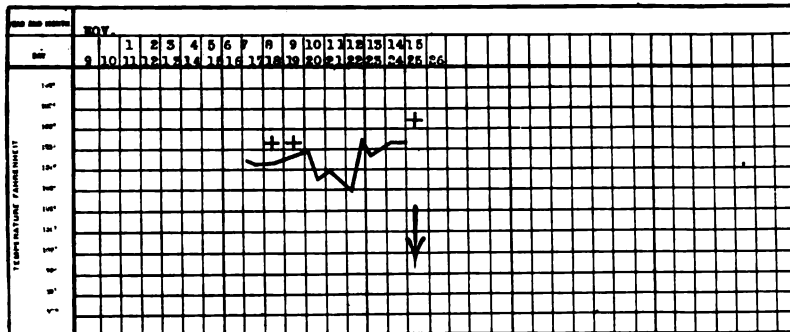
**December 5. Subacute or chronic case.**

December 10. Animal weak and emaciated.

**December 18. Animal moribund, bled and examined.**

Lungs, heart muscle, lymphatic glands, kidneys, and cecum show typical lesions.

## HOG No. 514. MICHIGAN (ROCHESTER) STRAIN OF VIRUS.



REMARKS: November 9-11. Rochester pigs were placed with this hog, which was normal.

November 25. Animal moribund, bled and examined.

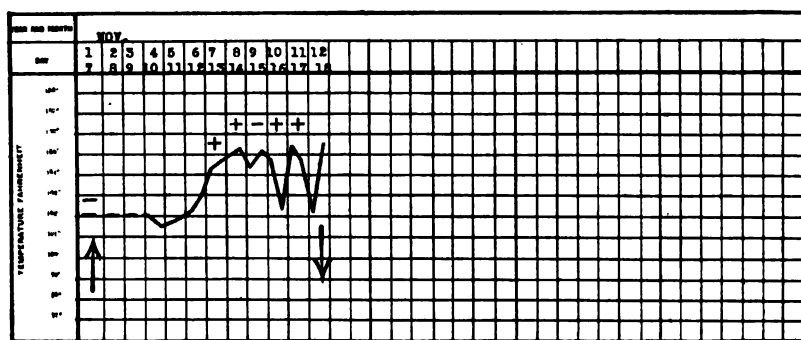
**Typical lesions, lymphatic glands, kidneys, lungs, and spleen.**

were received. Hog 514 had been kept for a period of several weeks in the pen for "susceptibles" previous to this time and was in a healthy condition. The animal was not otherwise exposed to the disease, but promptly developed cholera about six days after the Rochester pigs were placed with it. The blood of Hog 514 showed the presence of the spirochete on three different examinations, and autopsy revealed typical lesions of the disease.

*Indiana strain of virus.*—This virus was secured from Mr. Delplane and was originally supplied by Dr. R. A. Craig, who reported that this strain was secured by him in the field some time ago and did not represent the B.A.I. strain of virus.

Hog 501 was inoculated with 5 c.c. of Indiana I virus on November 7, after having made a dark field examination of the normal blood. On the seventh day after inocula-

HOG NO. 501. INDIANA I STRAIN OF VIRUS.



REMARKS: November 7. Inoculated with 5 c.c. of Indiana I virus.

November 12. 4 P.M. Animal showed symptoms.

November 18. Animal moribund, bled and examined.

Purple areas of discoloration on abdomen, nose, and ears. Subcutaneous blood extravasations. Lymphatic glands enlarged and hemorrhagic. Both kidneys petechiated and contain retention cysts. Hemorrhagic areas in heart muscle, lungs partially consolidated in both upper and lower lobes. Ulcers in cecum.

tion the spirochete was found in the blood of this animal, although symptoms, with the exception of a beginning rise of temperature, were not observed until the following day. Attention should be called to the fact that on the ninth day after inoculation no spirochetes could be found in the blood, but characteristic granules were present, while on the tenth day the spirochete was again found. This phenomenon, observed in other cases as well, suggests that the spirochete under observation represents one stage in the life cycle of the organisms, and that the granule formation is possibly an important factor in its development.

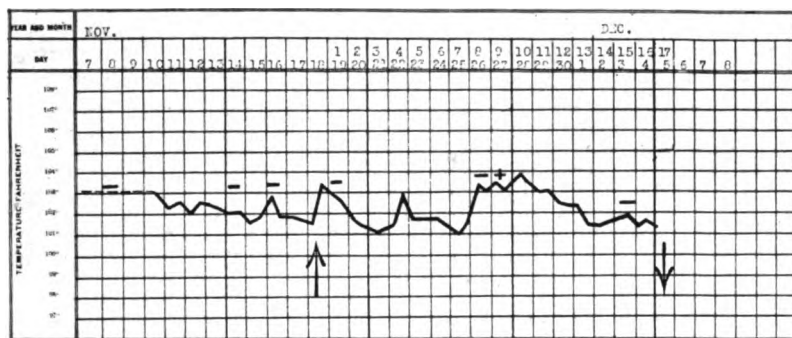
*California (Hall) strain of virus.*—Dr. Ivan C. Hall of California supplied this virus and the following history:

"We have just bled a hog originally injected with a virus obtained from one of our California towns. This virus was injected after being filtered and proven bacteria free, as it originally contained a pure culture of *B. cholera suis*.

"The hog became very sick about 10 days after and, appearing moribund, we bled him to death. The virus which I am sending you is some of this, after it has been diluted to one part in four and filtered through a Berkefeld filter."

Hog 502, which received the above strain of virus, had been kept for several days as a control on the isolation of the experiments and on the dark field examinations of the blood of normal and diseased hogs. Nine days after inoculation this animal exhibited mild symptoms of hog cholera, with temperature of 103.5° F. The spirochete was found in its blood. Fifteen days after inoculation its blood was free from spirochetes and characteristic granules. On the 17th day it was killed by large male Hog 400, and at autopsy no pathological lesions could be found.

HOG No. 502. CALIFORNIA (HALL) STRAIN OF VIRUS.



REMARKS: November 18. Inoculated with 15 c.c. California (Hall) virus (diluted 1-4) and filtered through Berkefeld.

November 26-30. Slight symptoms.

December 3. Animal apparently normal.

December 5. Animal killed by large male Hog No. 400. No lesions.

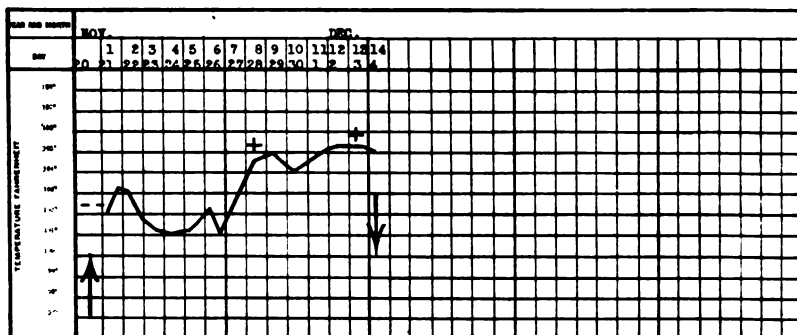
*Ohio (Pettigrew, Hazen, and Heinz) strains of virus.*—These viruses, obtained through the kindness of Dr. Paul Fischer and Dr. A. D. Fitzgerald, were from three different outbreaks and represented mixtures of blood samples from the number of pigs indicated on the labels. Ohio (Pettigrew), Ohio (Hazen), and Ohio (Heinz) represented mixtures of virulent blood from three, five, and six pigs respectively.

A dark field examination was made of the undiluted Ohio (Pettigrew) virus, as found in the original container, and one spirochete was observed.

The spirochetes were found in the blood of hogs inoculated with all three strains of Ohio virus, as shown on the following charts. The blood of Hog 521 (Ohio, Heinz strain) on the fifth day after inoculation contained relatively numerous spirochetes. On the seventh day none could be found, while on the eighth day the organism was readily found. By the eleventh day the clinical condition was much improved and the findings were negative, but the animal suffered a relapse and on the day of death the organisms were again present in the blood.

Hog 525, inoculated with the Ohio (Hazen) strain of virus, manifested symptoms on the fifth day after inoculation, and on that day the findings were positive. This animal is now fully recovered and no spirochetes are present in his blood.

## HOG No. 515. OHIO (PETTIGREW) STRAIN OF VIRUS.



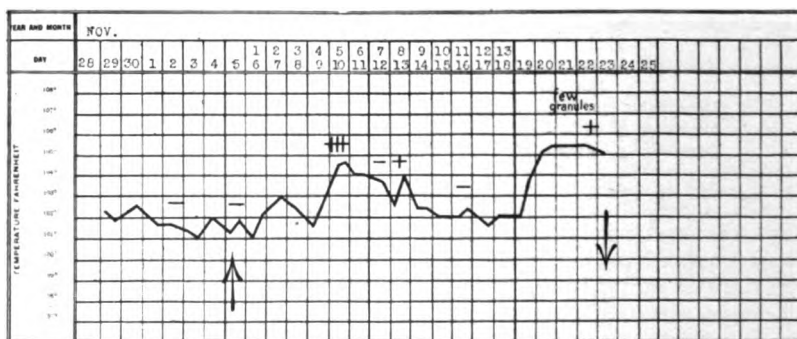
REMARKS: November 20. Inoculated with 15 c.c. filtered (Pettigrew) Ohio virus, diluted with normal saline 1-4.

November 29. Animal quite sick.

December 4. Animal moribund, bled and examined.

Spleen, kidneys, lymphatic glands, liver, lungs, and cecum show typical lesions.

## HOG No. 521. OHIO (HEINZ) STRAIN OF VIRUS.



REMARKS: December 2. Hog in good condition.

December 5. Inoculated with 15 c.c. Ohio (Heinz) virus, Berkefeld filtered and diluted 1-4.

December 10. Symptoms appeared.

December 15. Condition of animal improving.

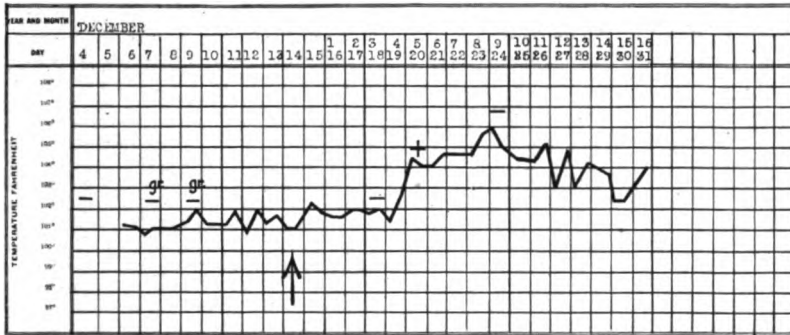
December 19. Animal suffered relapse.

December 23. Animal died.

Ventral surface of body deep purple color. Extravasations in subcutaneous tissue. Inguinal glands large and hemorrhagic. Lungs, both involved in all portions, highly congested, partially consolidated in small areas. Bronchial tubes filled with exudate. Heart, petechiae in auricles and the heart muscle. Both kidneys show small petechiae. Liver, passive congestion. Mesenteric and retroperitoneal and inguinal lymph glands enlarged and hemorrhagic. Cecum shows ulcers.

*Kansas I strain of virus.*—Professor L. D. Bushnell supplied this virus and described it as follows: "I sent you a couple of ounces of hog cholera blood, which was collected from an outbreak about a mile east of Manhattan, Kan., by Doctor Gingery. This animal was killed and showed typical lesions. It had a temperature of 108°. Twelve animals had died when the herd was vaccinated."

## Hog No. 525. OHIO (HAZEN) STRAIN OF VIRUS.

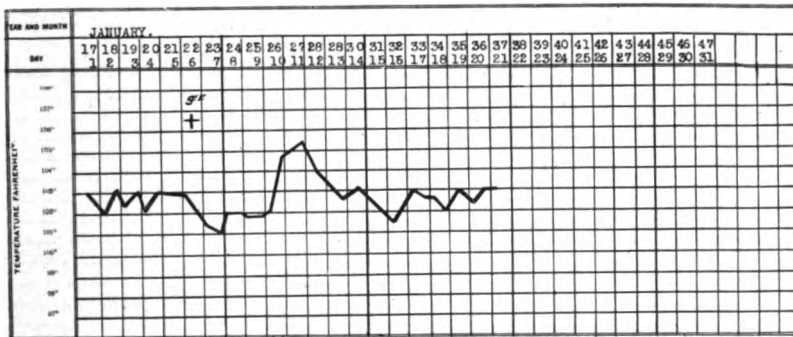


REMARKS: December 4. Susceptible pig; fine condition.

December 14. Inoculated 15 c.c. Ohio (Hazen) virus diluted 1-4 and Berkefeld filtered.

December 20. Anorexia, listless.

## Hog No. 525—Continued.



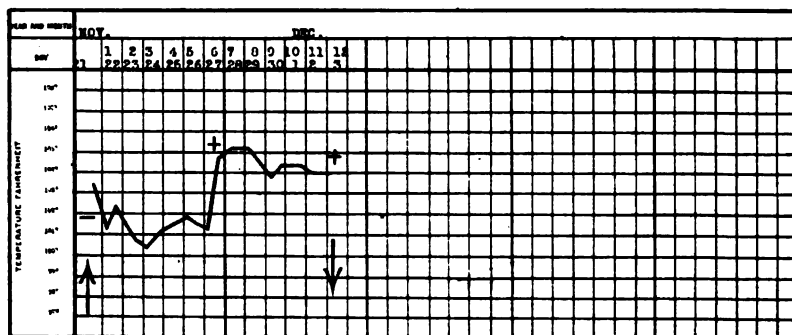
REMARKS: January 21. Animal fully recovered, released.

This virus, when received, was examined on the dark field. It was found to contain numerous granules, some bacteria, but no spirochetes were observed. The blood of Hog 516 inoculated with this virus showed the presence of the spirochete.

*Unknown strain of virus.*—On November 25 and 26, Hogs 517 and 518 were inoculated with California (Hall) and California (University) strains of virus respectively. Before inoculation, blood from these apparently normal animals was collected

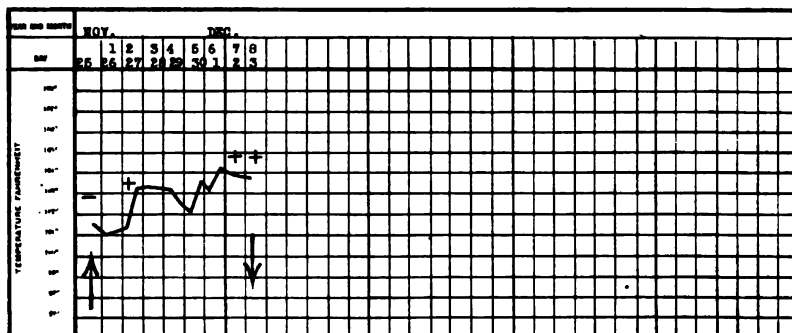
in sterile sodium citrate solution for control examination. In the case of Hog 518 the dark field examination of the assumed normal blood revealed the presence of the spirochete. Subsequent developments clearly indicated that these animals were in some way exposed to the disease before inoculation, as clinical evidences of the disease

Hog No. 516. KANSAS I STRAIN OF VIRUS.



REMARKS: November 21. Inoculated with 4 c.c. Berkefeld filtered Kansas I virus.  
 November 29. Animal very sick.  
 December 3. Animal moribund, bled and examined.  
 Typical lesions, marked in lymphatic glands, kidneys, cecum, spleen, and lungs.

Hog No. 517. UNKNOWN STRAIN OF VIRUS.



REMARKS: November 25. Inoculated with 15 c.c. California (Hall) virus, Berkefeld filtered and diluted 1-4.  
 November 26. Animal shows symptoms, evidently due to previous natural exposure.  
 December 3. Animal moribund, bled and examined.  
 Cecum, lymphatic glands, spleen, kidneys, and lungs show typical lesions.

were clearly present in both cases within a day or two following inoculation, an interval of too short duration to correspond with the incubation period which should follow the injection of virus. Moreover Hogs 517 and 518 were both in a moribund condition,

This strain of virus was injected into Hog 524 after having established three negative dark field examinations of his blood. As shown on the chart the spirochetes

TIME AND DATE		DAY							TIME	
TEMPERATURE	FRACTION	DAY							TIME	
		1	2	3	4	5	6	7	1	2
26	27	28	29	30	1	2	3			

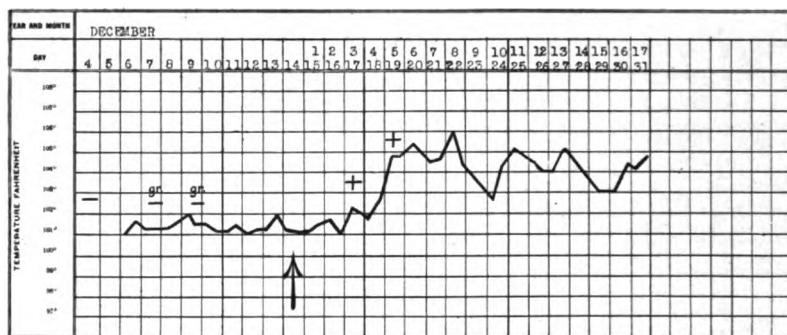
Small blood extravasations in muscular and subcutaneous tissue. Spleen slightly enlarged, dark gorged with blood. Capsules of both kidneys loosely adherent, and both kidneys filled with small urinary cysts. Both lungs show areas of consolidation. Inguinal and mesentery glands enlarged and hemorrhagic. Ulcers present in cecum.

On January 11, dark field examinations of the blood of two of these animals revealed the presence of the spirochetes. The temperatures of these two animals were each 103.8° F. and a tentative diagnosis of the chronic type of cholera was made. On January 13, three days after the receipt at the laboratory, the temperatures of seven of the 15 animals ranged between 104° and 105.5° F. and, from other symptoms, there was no doubt but that the diagnosis of cholera was correct. Investigation showed that on January 1, these animals were placed under such conditions that exposure to



hog cholera might result. As they were received at the laboratory on January 10 and symptoms definitely established by January 13, there existed a period of incubation corresponding to that usually present in the chronic or subacute types of cholera.

Hog No. 524. CALIFORNIA (UNIVERSITY) STRAIN OF VIRUS.

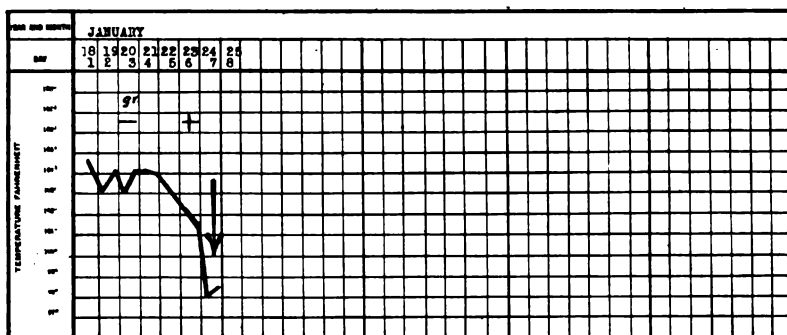


REMARKS: December 4. Susceptible pig; fine condition.

December 14. Inoculated 15 c.c. California (University) virus, Berkefeld filtered and diluted 1-4.

December 18. "Off feed." Symptoms appeared.

Hog No. 524—Continued.



REMARKS: January 8. Found dead.

Typical lesions, lungs, liver, kidney, spleen, cecum, inguinal glands, large intestine, and subcutaneous tissue.

These animals are under observation at the present time. A summary of the dark field findings is presented in the following table.

TABLE 1.  
MICHIGAN (ROCHESTER II) STRAIN OF VIRUS.

Hog	Number Dark Field Examinations to Date	Presence of Spiro- chete in Blood	Remarks
537.....	3	+++	Temp. range from 102 -104.8° F.
538.....	3	+++	" " " 101.2-104.1 "
539.....	3	++-	" " " 101.2-105.5 "
540.....	1	-	" " " 101.6-103.5 "
541.....	1	+	" " " 101.6-103.2 "
542.....	3	++-	" " " 102. -104.6 "
543.....	1	+	" " " 102. -103.5 "
544.....	2	++	" " " 102.4-105.5 "
545.....	1	-	" " " 101.8-103. "
546.....	1	-	" " " 101. -104.5 "
547.....	2	--	" " " 101.5-103. "
548.....	2	++	" " " 102. -105. "
550.....	1	-	" " " 101.5-103. "
551.....	1	-	" " " 102. -103.5 "
552.....	1	+	" " " 101.6-103.8 "

#### DARK FIELD EXAMINATIONS OF THE BLOOD OF NORMAL HOGS.

In addition to the control examinations which were made in practically all cases, as shown on the charts above, additional data relative to negative findings in normal blood have been secured.

TABLE 2.  
DARK FIELD EXAMINATIONS OF THE BLOOD OF HOGS NOT SUFFERING FROM HOG CHOLERA.

Hog	Number Dark Field Examinations	Spirochete Present or Absent	Condition of Animal
522.....	2	--	Normal
512.....	1	-	"
523.....	1	-	"
526.....	2	--	"
519.....	2	--	"
520.....	2	--	"
527.....	3	---	"
528.....	1	-	Infested with <i>Ascaroides</i>
529.....	2	--	" " "
530.....	1	-	" " "
531.....	1	-	" " "
532.....	1	-	" " "
533.....	1	-	" " "
534.....	1	-	" " "
535.....	1	-	" " "
536.....	1	-	" " "
400.....	1	-	Natural immune
553.....	2	--	Normal
554.....	2	--	"
555.....	2	--	"
556.....	2	--	"

A number of hogs, planned for material to be used in the work, became badly infested with intestinal parasites (chiefly *Ascaroides suella*) and were not utilized except for control blood examinations.

Some of these animals exhibited temperatures as high as 106° F.

at the time of the dark field examinations. The negative findings in these cases, therefore, present valuable data in that they show that the presence of the spirochete observed in the blood of animals suffering from cholera, does not depend merely upon a febrile condition of the blood.

Several immune hogs not included in the above data also served as donors of normal blood specimens. These additional negative findings in the blood of hogs not infected with hog cholera are shown in Table 2.

#### HORSE SERUM VIRUS PHENOMENON.

Horse serum virus may be produced by securing blood from a horse one to two hours after the animal has received an intravenous injection of approximately 150 c.c. of virulent hog cholera serum. The character of this material and the results of experiments, which demonstrate that it is not a mere dilution of hog cholera serum, have been reported in former publications.<sup>1</sup> It may be concisely stated that hog cholera virus undergoes some unexplainable process of activation after approximately one hour's residence in the circulatory system of the horse.

On October 25 the blood of Horse 1 was examined on the dark field. Nothing other than erythrocytes, leukocytes, and some blood dust was noted. At 1:45 P.M. on October 25, Horse 1 was given an intravenous injection (jugular vein) of 150 c.c. B.A.I. strain of virus. A specimen of this hog cholera serum was examined on the dark field and no spirochetes were found, although the material did contain many characteristic granules. The animal was led from the stocks and immediately became groggy and weak, the pulse was weak, respiration labored, eye dull and listless, and peristalsis violently active. The animal became covered with a profuse cold perspiration and frequent watery evacuations followed. Three-fourths of an hour after the injection of the horse it was bled under aseptic conditions and the blood examined by dark field illumination. The spirochetes were found with ease, six organisms being found in a few minutes. Blood drawn from the horse one and three-fourths hours after injection also contained the spirochete when examined on the dark field. The one and three-fourths hours virus also contained a marked increase in the number of granules. A specimen of the blood of Horse 1 drawn two and three-fourths hours after injection upon careful dark field examination revealed only granules and refractive bodies—no spirochetes could be found.

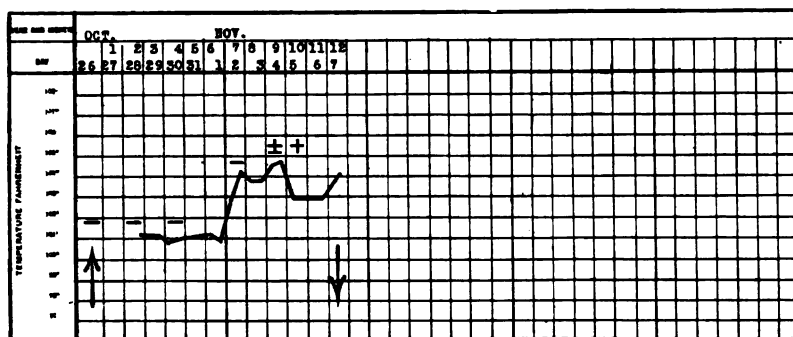
The virulence of the three-fourths-hour horse serum virus which the above animal yielded was tested by the inoculation of Hog 4501. The result of this animal inoculation conformed with extensive data previously reported, in that the hog became the

<sup>1</sup> King and Wilson, *Bull. Kansas Exp. Sta.*, 1910, No. 171. *Jour. Infect. Dis.*, 1912, 11, p. 441; *Ztschr. f. Immunittsf. u. Exp. Ther.*, 1913, 16, p. 367.

subject of a more acute type of cholera after a relatively short period of incubation, as compared with the average case following the injection of hog cholera serum itself.

On November 22, Horse 2 was utilized in duplicating the above experiment, after establishing a normal condition of the blood in so far as absence of spirochetes was concerned. This animal was injected intravenously with 180 c.c. of B.A.I. and Indiana I strains of virus mixed. The symptoms were of the usual character, but were more retarded and less pronounced than those recorded in the first experiment with Horse 1. In our experience,<sup>1</sup> more pronounced reactions following injection and more uniform horse serum virus result, when the animals have been subjected to one or more repeated injections with the hog cholera virus, a condition in all probability not depending upon

HOG No. 4501. B.A.I. STRAIN OF VIRUS. HORSE SERUM VIRUS.



REMARKS: October 26. Inoculated with 4 c.c. activated horse serum virus.

November 2. Symptoms appeared.

November 7. Animal found dead and examined.

Many subcutaneous blood extravasations. Muscle of right ventricle contains blood extravasations. Lower lobe left lung consolidated. Areas of red hepatization in upper lobes of both lungs. Spleen soft, dark, and gorged with blood. Kidneys show numerous petechiae. Large ulcer near ileo cecal valve. Extensive areas of congestion in intestinal mucosa. Lymphatic glands enlarged and hemorrhagic.

anaphylaxis as shown by previous results. Horse 1, above, had been repeatedly used on the work; Horse 2 had never been subjected to the treatment before.

The three-fourths-hour horse serum virus of Horse 2 showed the spirochetes on the dark field. In the two-hour virus only one spirochete could be found.

The above data must be verified by repeated experiments. However, assuming that the spirochete under observation, in different stages of its life cycle, might represent the etiological factor in hog cholera, it would not be impossible to explain the horse serum virus phenomenon by the results obtained above.

<sup>1</sup> Grateful acknowledgment is made to Dr. R. H. Wilson, who for several years has been associated with the senior author in this work, and who rendered valuable assistance in carrying out the horse serum virus experiments included in the present investigation.

## SUMMARY.

The results may be summarized as follows:

1. Dark field examinations of the blood of 48 hogs, all normal, excepting some which were infested with intestinal parasites, have revealed no spirochetes. All of these specimens have been relatively free from granules, with the exception of some instances, when it was usually possible to determine that the granules were liberated from crushed leukocytes.
2. The examination of the blood of all hogs—40 animals—which were suffering from hog cholera, revealed the presence of a spirochete. All specimens of blood from these animals, during the height of the disease, contained characteristic granules.
3. Negative dark field findings followed positive findings in the case of six hogs which recovered from the disease.
4. The blood of two naturally immune hogs was free from spirochetes and granules.
5. The spirochetes and granules have been observed in hogs infected with 12 different strains of virus, as follows: B.A.I., Michigan (Demerick), Michigan (Rochester I), Indiana I, California (Hall), Ohio (Pettigrew), Ohio (Hazen), Ohio (Heinz), Kansas I, Unknown, California (University), and Michigan (Rochester II).
6. Hog cholera was diagnosed in two small herds of hogs after receipt of the animals at the laboratory, by means of the dark field examination of the blood. In other instances, positive dark field findings were secured a few hours before any symptoms appeared.
7. The spirochetes were found with less difficulty in the blood of hogs suffering from the acute form of the disease.
8. In two experiments the spirochetes were demonstrated in horse serum virus.
9. Spirochetes are uniformly demonstrable in the intestinal ulcers of hogs dead from cholera.

## CONCLUSIONS.

Without cultures with which to inoculate susceptible animals it is, of course, impossible at this time to definitely ascribe to the spirochete under observation any pathogenic properties or etiologi-

cal significance. Nevertheless, careful consideration should be given to the results which are consistent and uniform throughout, and which, in some particulars, strongly suggest the relationship of this spirochete to hog cholera, as a causative factor. As this organism has not been previously reported and described the name *Spirochaeta suis* may properly be suggested.

## TUBERCULOSIS OF THE STOMACH WITH EXTENSIVE TUBERCULOUS LYMPHANGITIS.\*

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In most of the articles on tuberculosis of the stomach, two forms, the ulcerative and the hematogenous miliary, are discussed. A third form, the tuberculous lymphangitis, frequently met with in the intestine, is mentioned in few and then only incidentally to accounts of the ulcerative type of lesion. That such a lesion does occur in the stomach and that it may possess features distinct and unlike either of the two varieties, is borne out by the conditions described in the following account.

It concerns a laboring man, 45 years old. The anatomic diagnosis was ulcerative tuberculosis of both apices, and tuberculous bronchopneumonia; caseous tuberculosis of the tracheobronchial lymph glands; disseminated nodular tuberculosis of the inner coat of the stomach; ulcerative tuberculous colitis; emaciation and anemia; brown atrophy of the heart; external fibrous pericarditis; passive hyperemia and atrophy of the liver; bilateral obliterative pleuritis; fibrous adhesions between the descending duodenum and the under surface of the liver. The emaciation was extreme. The body, which was of average height, weighed 64 pounds. The upper arm could be spanned by the thumb and ring finger of one hand, the greatest circumference of the thigh was 27.5 cm.

The tuberculosis of the stomach has formed numerous rather firm elevations of the lining, averaging two to four millimeters in height, on the posterior and anterior wall, but chiefly on the posterior along the lesser curvature (see Fig. 1). They are reddish, from a few millimeters to one centimeter in length, many of them rather oblong. In one or two places there are suggestions of superficial erosion caused possibly by rough handling in removal. Some of these ridges are two to three centimeters in length and continuous with elevations which are seen and felt externally.

The tuberculous colitis consists of four large ulcers with rounded regions of thickening similar to those observed in the stomach.

The lesions in the stomach are seen very distinctly, especially by holding the specimen against the light, when at once a certain definiteness of grouping and distribution is apparent. In order to ascertain the relationship of these lesions, the stomach after being made still more translucent by museum fluids was examined by transmitted light and the general correspondence in direction and location between the ridges and blood vessels is illustrated by Fig. 2. The ridges follow the course of the lymph channels as they are described and illustrated by Sappey.<sup>1</sup> Cunéo

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<sup>1</sup> *Anatomie, Physiologie, Pathologie des vaisseaux lymphatiques*, Paris, 1874.

and Delamare,<sup>1</sup> Poirier and Charpy,<sup>2</sup> Most,<sup>3</sup> Jamieson and Dobson<sup>4</sup> (see Fig. 3), and Comolli.<sup>5</sup>

It is apparent that the ridges are arranged chiefly toward and along the lesser curvature, involving the coronary group of lymphatics. The splenic group is represented to some extent, while the hepatic or right gastro-epiploic group is scarcely in evidence.

The tuberculous nature as well as the relation to the lymph channels of these



FIG. 1.—The ridges and nodular or polypous elevations are grouped chiefly about the lesser curvature. See Fig. 2.

lesions was confirmed by the microscopic examination. Tubercle bacilli are present in large numbers, contrary to the usual reports dealing with tuberculosis of the stomach.

In all respects the structure is typical of tuberculous lesions. Giant cells are found in nearly every section. The infiltration is enormous in those portions where the mucosa is raised to form the ridges; it is especially dense in the mucosa itself where it reaches very close to the surface. Rows of lymphocytes extend along the muscularis mucosae and in the submucosa. In many places they are found just without thin-

<sup>1</sup> *Jour. de l'anat. et physiol.*, 1900, 36, p. 393.

<sup>2</sup> *Traité de l'anatomie humaine*, Paris, 1901, 2; also in English as *Special Study of the Lymphatics in Different Parts of the Body*, by Poirier and Cunéo, Chicago, 1904.

<sup>3</sup> *Arch. f. klin. Chir.*, 1899, 59, p. 175.

<sup>4</sup> *Lancet*, 1907, 1, p. 1061.

<sup>5</sup> *Arch. Ital. di Anat. e di Embr.*, 1911, 10, p. 103.



walled lymph channels which do not contain red blood corpuscles. They are as large as the veins but possess thinner walls. In a few places the collections of lymphocytes within them resemble beginning intimal tubercle formation.

The lymph nodes are numerous, and some of them are apparently hyperplastic. Some are deeply stained; others have large germinal centers.

The muscularis and the serosa are intact.

The nearest approach to similar conditions which I have been able to find in the literature of tuberculosis of the stomach is in



FIG. 2.—The dotted line A represents the location of the lesser curvature, the dotted line B, that of the greater curvature, the many small curved lines, the location of the blood vessels. This is one-half the natural size and the original was made by studying the location of the blood vessels in the Kaiserling (museum) specimen with transmitted light. The general correspondence between the location and direction of the tuberculous lesions and blood vessels is obvious.

the article by Przewoski.<sup>1</sup> He reports five cases of tuberculosis of the stomach, all of the ulcerative type. In his description of the first, in which there were 22 ulcers, he says:

"Around the ulcers the submucosa is distinctly thickened in the form of whitish ridges, two and more centimeters long, extending in all directions or only in some. Over these thickenings of the submucosa the mucosa is less movable and the wall much less transparent. The muscularis under the floor of the ulcers is somewhat thickened and traversed by narrow whitish ridges and some small grayish-white nodules can be seen in several places in the serosa." The microscope discloses "proliferation and leuko-

<sup>1</sup> *Virchow's Arch.*, 1902, 167, p. 424.

cytic infiltration along thin-walled spaces accompanying the arteries and veins of the submucosa. Quite often rather broad spaces lined with thickened endothelium can be seen in the center or alongside these rows of leukocytic infiltration. Along these spaces and within their walls the infiltration presents itself here and there in the form of small nodules showing epithelioid cells, giant cells and Koch's bacilli," and the writer concludes: "Hence this is a lymphangitis tuberculosa around the tuberculous ulcers of the stomach. A leukocytic infiltration of this type along the blood and lymph vessels has also produced the whitish lines in the muscularis under the floor of the ulcers."

Of the third case he says: "The only striking feature in this case is the extensive participation of the lymph vessels of the submucosa in the tuberculous inflammation."

In the fourth case in which there are two ulcers, two centimeters apart from each other, situated on the posterior wall, four and three centimeters in diameter, he states: "The submucosa between the ulcers is visibly thickened. Besides this there is a distinct thickening in the form of ridges surrounding the larger ulcer at a distance of one to two and more centimeters."

Przewoski finally compares this type of gastric ulcers with the tuberculous ulcers commonly found in the intestine, and as one of the differential points he mentions the feature which I have singled out from his description, namely the much more marked involvement of the submucosa in the tuberculous inflammation of the stomach. Whether, however, this is a frequent occurrence in the stomach, he admits he is not prepared to say.

In addition to this very definite description of a tuberculous lymphangitis in Przewoski's article we may find, here and there, casual remarks which suggest the existence of a lymphangitis. For example, Schwalbe<sup>1</sup> mentions a "moderate round-cell infiltration in the lymph spaces of the tunica muscularis"; Renon and Verliac,<sup>2</sup> "distended and caseous lymphatics especially in the muscularis"; and Ricard and Chevrier<sup>3</sup> "gray and yellowish lines and dots in the floor of the ulcers."

The few statements quoted indicate that tuberculous lymphangitis is at least less commonly noted in the stomach than in the bowel. However, although there is little mention of it in the published accounts of tuberculosis of the stomach, they do contain abundant proof of the rôle played in the morbid anatomy of the lesions by the well-known inherent disposition of this inflammatory process to follow lymph channels. I venture to suggest that the plan of the gastric lymphatic system (see Fig. 3) is, in general, not

<sup>1</sup> *Virchow's Arch.*, 1889, 117, p. 316.

<sup>2</sup> *Bull. et mêm. Soc. mêt. d. hôp. de Par.*, 1907, 4, p. 111.

<sup>3</sup> *Rev. de chir.*, 1905, 31, p. 74, p. 557, p. 736.

as familiar to us as that of the intestine, which is a regular and uniform one, that therefore certain features, which in the intestine have been connected with the lymphatics, have not been interpreted or discussed in the same manner. It is conceivable that in descriptions of small ulcers being dispersed here and there or continued in rows from the large ulcers, the distribution of the minor lesions may have followed the course of lymph vessels. In the arrangement of these minute ulcers we can perhaps discover a certain resemblance with the rosary-like extension of tubercles so frequently observed

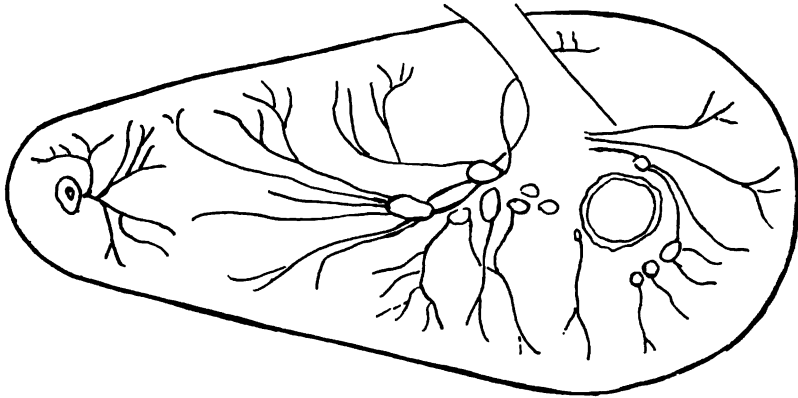


FIG. 3.—The lymph glands and lymph vessels about the cardia. From Jamieson and Dobson.

in the intestine. The extensive ulceration described by Eppinger<sup>1</sup> admits of such an association of the lesions with the lymph channels. In one of his cases there were, on the inner surface, innumerable losses of substance, up to 1.5 cm. diameter, forming whitish rows or ridges along the lesser curvature toward the pylorus, on the posterior and the anterior wall.

In another case there were everywhere losses of substance, arranged in rows, almost throughout the size of a pea. Besides these rows of ulcers innumerable, irregularly distributed ulcers of various sizes were scattered over the walls of the stomach.

Rosset<sup>2</sup> reports an interesting case. Close below the cardia there is an almost round ulcer, about the size of a penny. Right

<sup>1</sup> *Prag. med. Wchnschr.*, 1881, 6, p. 501.

<sup>2</sup> *Ueber einen Fall von tuberkulösem Magengeschwür. Mit besonderer Berücksichtigung der Genese. I.D., Freiburg i. B.*, 1903.

beside this begins a large, irregularly shaped, more oblong loss of substance whose development from smaller, round ulcers can still be distinctly recognized; it measures about 5.5 cm. in length and 5-12 mm. in width. At the lower end of this oblong ulcer, separated from it by a bridge of normal mucosa about eight millimeters broad, a smaller, almost round ulcer, one centimeter in diameter, is found.

Litten's case<sup>1</sup> presents on the anterior wall of the stomach in the region of the lesser curvature an elliptic ulcer, about 4.2 cm. long and 3.3 cm. broad, the long axis of which corresponds to the direction of the curvature.

Other reports of tuberculosis of the stomach have this in common that the ulcers are oblong and extend along the lesser curvature. May we not assume that the confluence of small ulcers like those in Eppinger's cases along lymph channels, analogous to the familiar processes in the intestine, determined the form and direction of the lesions he described?

A further explanation of the scarcity of reports of tuberculous lymphangitis in the stomach is possibly due to the greater thickness of the wall of the stomach as compared with the intestine and the consequent failure to recognize the relationship to blood vessels so easily perceived in the bowel.

No doubt, too, the microscopic examination which would reveal a lymphangitis is often made simply to confirm the gross anatomic diagnosis.

The rarity of tuberculosis of the stomach as compared with its frequency in the bowel has resulted in studies directed to ascertain the nature of the insusceptibility generally possessed by the stomach and of its vulnerability when involved. Of these studies the following deserve brief mention. Rousseff<sup>2</sup> found nontuberculous alterations in the lining of the stomach were more frequent when there was an intestinal tuberculosis secondary to one in the lungs than when there was not.

A number of writers<sup>3</sup> have discussed the relation of lymph

<sup>1</sup> *Virchow's Arch.*, 1876, 67, p. 615.

<sup>2</sup> *Des rapports qui existent entre la tuberculose intestinale et les altérations stomacales dans la tuberculose pulmonaire*, Thèse de Genève, 1890, cited by Zahn, *München. med. Wchnschr.*, 1902, 49, p. 49.

<sup>3</sup> Barbacci, *Lo Sperimentale*, 1890, 65, p. 475; Przewoski, *op. cit.*; Dobrowolski, *Zeigler's Beitr.*, 1894, 4, p. 43; Wilms, *Centralbl. f. allg. Path. u. path. Anat.*, 1897, 8, p. 783.

nodes in the mucosa to the tuberculous process. Granted that they may be regarded as the starting-point of the latter, the question still remains, how the tubercle bacilli get to these small masses of lymphoid tissue when the first involvement begins.

There are four possible ways for the tubercle bacillus to enter the gastric mucosa: (1) by direct infection of the mucosa, (2) by a hematogenous invasion, (3) by way of the lymphatics, (4) by direct propagation from a tuberculous focus to the serosa with which it is in close contact. The first mode of infection which has been the subject of a number of thorough investigations will not be discussed here. It cannot absolutely be excluded in the instance studied. We have no reason to believe that the mucosa was entirely normal. A degree of gastritis seems to be the rule in the terminal stage of pulmonary tuberculosis and possibly existed here too. Whether there were other predisposing factors for catarrhal conditions, such as alcohol, is unknown. According to Rousseff's observations, already mentioned, that intestinal tuberculosis without exception is always accompanied by distinct structural changes in the gastric mucosa, the presence of four large ulcers in the colon in our case speaks for such changes.

As for the hematogenous mode of invasion, Arloing<sup>1</sup> believes that it should occupy the first rank in considerations of the etiology of gastric tuberculosis. He has written exhaustively on the subject and he supports his views by conclusions from considerable experimental work by himself. Wilms<sup>2</sup> and Simmonds<sup>3</sup> express the opinion that the miliary hematogenous type of gastric tuberculosis is of much more frequent occurrence than is generally believed.

Before I pass to the third mode of infection which will receive the chief consideration, the last type—that by propagation from a neighboring tuberculous focus to the stomach—may be dismissed briefly. The anatomical conditions of the stomach are such that we must consider cases which seem to speak for such an infection as extreme exceptions. Possibly even these cannot be accepted

<sup>1</sup> *Des ulcérations tuberculeuses de l'estomac* (Étude clinique, expérimentale et anatomo-pathologique), Lyon, 1902.

<sup>2</sup> *Centralbl. f. allg. Path. u. path. Anat.*, 1897, 8, p. 783.

<sup>3</sup> *München. med. Wchnschr.*, 1900, 47, p. 317.

without reserve, since we see that even a case like that which Chiari<sup>1</sup> reports where caseous masses from a much broken down lymph gland extended through a perforating ulcer into the lumen of the stomach admits of another plausible interpretation as we shall see later.

Concerning the third mode of infection—that by way of the lymph channels—very little attention has been given this type throughout the literature on tuberculosis of the stomach. It is in general admitted as a possibility, even a probability, but is rarely discussed.

In textbooks and treatises on tuberculosis, special emphasis is laid on the lymphogenic origin and spread of tuberculosis in general. Orth<sup>2</sup> writes: "It has long been noticed by observers, that the tubercles in the most diverse organs preferably locate along the lymph vessels, and though it is going too far to say that tuberculosis is a lymphangitis, nevertheless it is certain that a lymphangitis tuberculosa in the small branches belongs to the most frequent changes. They are best seen in the lymph vessels of the bowel, surrounding a tuberculous ulcer of the mucosa, where the subserous lymph vessels are often beset with small gray tubercles for considerable stretches."

Klebs<sup>3</sup> refers to it as follows: "He who has not convinced himself by personal investigation that tuberculosis is propagated exclusively by way of the lymph channels," etc., and "the infection spreads by continuity, preferably by way of the lymph vessels, and only later by way of the blood vessels." Rindfleisch came to similar conclusions. "From tuberculous ulcers in the intestine rows of nodules follow the course of the lymph vessels, often to the next glands; a lymphangitis tuberculosa this might be called."

It is interesting to note here a statement made by Dürck and Oberndorfer:<sup>4</sup> "While the spread of tuberculosis by way of the lymph channels plays a great rôle, it is remarkable, however, that beside the frequent morbid involvement of the lymph glands the lymph vessels themselves are so rarely diseased, so that apart from

<sup>1</sup> *Wien. med. Wchnschr.*, 1878, 24, p. 650.

<sup>2</sup> *Lehrbuch der speciellen pathologischen Anatomie*, Berlin, 1887, 1, p. 275.

<sup>3</sup> *Virchow's Arch.*, 1868, 44, p. 242.

<sup>4</sup> *Ergebn. der. allg. Path. u. path. Anat.*, 1899, 6, p. 354.

the tuberculosis of the thoracic duct, described by Benda, cases of lymphangitis tuberculosa belong to the great rarities."

Ruge<sup>1</sup> opposes retrograde infection in lymph channels in the following words: "Now, a lymphatic invasion of bacilli into the gastric mucosa would have to take place in a direction opposite to the normally coursing lymph stream. With the non-motility of the tubercle bacillus such a supposition would encounter considerable difficulties. The same is true of the subsidiary hypothesis of an eventual retrograde lymph current in the corresponding vessels." On the other hand the mechanical difficulties to retrograde metastases cannot be great. Cunéo and Delamare<sup>2</sup> write: "Inasmuch as the lymph glands become actually infected, through embolism, by the intermediary of certain of their afferents, their other afferents can be invaded by retrograde thrombosis and by this mechanism infect the organ, in which they have their origin." Orth<sup>3</sup> states that "obstruction to the lymph stream with subsequent varicosity of the lymph vessels in the intestine or toward the mesenteric glands is not rare." Perhaps more to the point since it deals with actual conditions in the stomach are the evidences that such a mode of dissemination does take place in tuberculosis of the stomach contained in the report by Rosset<sup>4</sup> concerning a man, 80 years old, with emphysema, a beginning pulmonary edema, slaty induration of the lung with fibrous nodules. There were caseous bronchial and retroperitoneal lymph glands behind the stomach. Some of the mesenteric glands were as large as a bean and showed a black mass on the cut surface. In several places of the intestine, slight ulcerations, the size of a pinhead, and in one place a somewhat larger, yellow, scarred spot were found. In the stomach there were three tuberculous ulcers; close below the cardia a nearly round ulcer, the size of a penny, in the midst of a markedly injected mucosa; close behind this, a larger, irregular, more oblong ulcer, 5.5 cm. long, 5-12 mm. broad, in which the development from smaller round ulcers could still be recognized distinctly; at some distance from its lower end a smaller, nearly round ulcer, one centimeter in diameter, separated from the oblong ulceration by a bridge of normal

<sup>1</sup> *Beitr. z. Klin. d. Tuberkulose*, 1905, 3, p. 191.

<sup>2</sup> *Jour. de l'anal. et physiol.*, 1900, 36, p. 393.

<sup>3</sup> *Op. cit.*

<sup>4</sup> *Op. cit.*

mucosa, measuring about eight millimeters. The rest of the mucosal surface was perfectly normal. Several lymph glands in the neighborhood of the cardia and behind the stomach were enlarged and totally caseated.

As to the origin of these ulcers, Rosset argues as follows: With the very slight extent of the intestinal tuberculosis the possibility of propagation of the infection from the intestine to the stomach may be excluded. A direct infection of the mucous membrane by ingesta containing tubercle bacilli is very improbable; tuberculous ulcers are hardly ever met with in just the pars cardiaca. The complete caseation of the retrogastric lymph glands shows that the tuberculosis in them probably existed longer than the tuberculous ulceration in the stomach. Moreover, not the least manifestations of tuberculosis of a fresh nature were found in the lungs, so that an infection by tubercle bacilli from the lungs cannot be presumed. There remains the supposition that the invasion was from the retrogastric lymph glands. A perforation from without could not be demonstrated macroscopically and the microscopic examination showed simply changes that involved the inner layers and at the highest reached the outer layer of the muscularis; the caseous foci in the submucosa were always separated by healthy normal tissue from the lymph glands situated below the serosa. There was nowhere communication of any of these lymph glands with the foci in the submucosa and muscularis.

Rosset believes therefore that in consequence of the tuberculous process in the retrogastric lymph glands and the anatomical changes resulting from this, there was stasis of the lymph stream, producing a retrograde current which carried the tubercle bacilli into the stomach wall. The primary infection was in the lymph glands at the hilus of the left lung. From them the infection was carried to the retrogastric lymph glands, which are connected with the mediastinal lymph glands by lymph vessels. In these retrogastric glands the tuberculous process brought about changes in the circulatory system whereby the normal outflow of the lymph from the gastric mucosa was hindered and a reversed flow set in. From the resulting necrosis and ulcerations large masses of tuberculous material passed into the stomach and from there into the intestine



where they produced the small ulcerations which seemed to be quite recent.

Rosset expresses the view that Chiari's case, which I have mentioned, might possibly be considered as a more advanced stage of his own case; that, if his patient had lived longer, caseation would have taken larger dimensions and finally healthy portions which separated the diseased areas would have melted down, until at last in one place, all the layers of the stomach wall would have undergone dissolution in the tuberculous process.

Also the third case in Hamilton's<sup>1</sup> report is worthy of comparison. "The stomach was adherent to the transverse colon, the pancreas, and to the mass of peripancreatic lymph glands. The serous coat was covered with small and larger caseous tubercles. On the posterior wall and part of the lesser curvature there was an ulcer three centimeters by two centimeters. Directly behind this ulcer was a caseous lymph gland, so closely adherent to the stomach wall that it was impossible to say whether or not it formed the floor of the ulcer. The microscopic examination proved, however, that the process in the stomach was quite independent in its origin, as the still intact muscular wall could be traced along the whole extent of the ulcer between it and the caseous gland."

I have quoted Rosset's case in detail, because his very plausible interpretation of the conditions that were present, and his way of tracing the infection, seem to me in part applicable to the conditions I have studied.

In regard to extension of infections from the thorax into the abdomen and vice versa, Küttner's<sup>2</sup> studies of the lymphatic system of the diaphragm have demonstrated the presence of numerous perforating lymph vessels from the thorax into the abdominal cavity and vice versa, and what apparently is a retrograde current is in reality normally directed. Such expressions as "retrograde" and "against the current" should be used cautiously, for, as Küttner shows, the determination of the direction of the current in a lymph vessel is often only possible by a direct observation of the injection of this vessel.

The perforating vessels of the diaphragm pass to the anterior and

<sup>1</sup> *Johns Hopkins Hosp. Bull.*, 1897, 8, p. 75.

<sup>2</sup> *Beitr. z. klin. Chir.*, 1903, 40, p. 136.

posterior mediastinal lymph glands on the one side, and to the parapancreatic, paraœeliac, and paraaortic glands on the other. They are more numerous than those which remain in the corresponding cavity. The lymph vessels of one half of the diaphragm do not communicate with those of the other half; on the other hand there is a very extensive communication between the lymphatic system of the pleural and the peritoneal surface of the same half of the diaphragm. The vessels which carry lymph from the diaphragmatic peritoneum to glands within the abdominal cavity, also take up the perforating channels from the diaphragmatic pleura. All the large lymph trunks of the diaphragm carry both pleural and peritoneal lymph. Such a system, and a perfect arrangement, both mechanical and anatomical, for the movement of the lymph within the layers of the diaphragm make this partitional wall between the two large body cavities particularly suitable to the spread of morbid processes from one to the other. Tendeloo<sup>1</sup> has emphasized the frequency in tuberculosis of adhesions between one or both lungs and the diaphragm and their importance in favoring the conveyance of infectious material along a seemingly unusual direction; for they result in conditions which necessarily affect the two main factors in the promotion of the diaphragmatic lymph, namely the difference of pressure between the thoracic and the abdominal cavity and the alternating state of contraction of the diaphragmatic musculatum, whereby now suction, now pressure is effected. The perforating lymph vessels of the diaphragm in all probability play an important rôle in metastases of infectious processes from the thoracic into the abdominal cavity, and vice versa.

From these considerations it is easy to understand how, in the case I have presented, infectious material could be carried from the lungs or also from the intestinal ulcers to the para- and prae-aortic glands and be deposited there, how from these glands it could find its way into the perigastric glands and how there in the melting-down process an obstruction could be offered to the normal outflow of the lymph from the stomach with the result of a backward flow from the infected station and importation of tubercle bacilli into

<sup>1</sup> *München med. Wchnschr.*, 1904, 35, p. 1537.

the stomach, producing there a lymphangitis which designed figures on the stomach wall that in a measure reproduce the picture of the lymphatic system in the corresponding area.

Can a direct infection of the mucosa in this case be excluded? Not absolutely. But I am inclined to believe that the majority of all cases of tuberculosis of the stomach, which clearly are not a hematogenous infection, have a lymphogenous origin, and that a direct infection of the mucosa should be admitted only in those cases where by complete destruction of the protecting epithelium, as for example, Breus'<sup>1</sup> case, or by some extraordinary coincidence as cancer in the stomach<sup>2</sup> the mucosa was made an exceptionally favorable culture-ground for the tubercle bacillus.

#### SUMMARY.

In studying the case discussed here, and in reading literature on tuberculosis of the stomach, my attention has been directed to two main points, brought to the front by the character of the lesion, which I have endeavored to make clear in this paper: (1) to establish the fact that a tuberculous lymphangitis may occur in the stomach which is not associated with ulcers as the starting-point, but which, in all probability, would ultimately lead to the development of ulcers of the various types known to us from the literature; (2) to give the lymphogenous origin in tuberculosis of the stomach a more positive and a more general consideration than has hitherto been done. Though in my case a direct infection of the gastric mucosa cannot be absolutely excluded, the ensemble of the conditions in this case, the fact of the general tendency of tuberculous processes to spread along lymph channels, and conclusions drawn from the literature on this subject lead me to assume the lymphogenous nature of the infection not only in my case, but in the larger number of cases of tuberculosis of the stomach.

<sup>1</sup> *Wien. med. Wchnschr.*, 1878, 28, p. 11.

<sup>2</sup> See Simmonds, *op. cit.*

## THE BEHAVIOR OF THE TUBERCLE BACILLUS TOWARD FAT-DYES.\*

### STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. V.

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The difficulty of staining the tubercle bacillus, together with its subsequent resistance to decolorization by alcohol or by acid, first recognized by Ehrlich, has been attributed to material of a fatty nature, present especially in the outer envelope. Of those who have engaged in the investigation of this acid-fast material, Bulloch and Macleod, in their résumé of previous work, state that De Schweinitz and Dorset and Klebs consider it fat—the former workers having demonstrated the existence of palmitic, arachidic, and lauric acids—while Ruppel found three kinds of fatty substance, differentiable on the basis of the solvent used for extraction, and containing fatty acids, esters, and higher alcohols; Aronson obtained by analysis from the acidified alcohol-ether extract, free fatty acid and a waxy substance, probably an alcohol. Kresling, using chloroform as an extractive, decided that the “fat” was a mixture of a neutral fat, free fatty acid, esters, and higher alcohols (lecithin and cholesterol). Bulloch and Macleod<sup>1</sup> themselves demonstrated that the alcohol and acid-resistance were due to the presence of an acid-fast substance, chemically an alcohol, though non-acid-proof materials decomposable into fatty acids (notably oleic, isocetinic, myristinic, and lauric), together with lipochromes, to which cultures of tubercle bacilli owe their color, were also present.

J. Lorrain Smith<sup>2</sup> holds that the resistance of stained fats to acid decolorization is “another argument in favor of the accepted doctrine that the ‘acid-fastness’ of tubercle bacilli is due to the presence of fat in their capsules,” and states that ordinary bacteria

\* Received for publication February 14, 1913.

<sup>1</sup> *Jour. Hyg.*, 1904, 4, p. 1.

<sup>2</sup> *Jour. Path. and Bacteriol.*, 1906, 11, p. 415.

acquire "acid-proof" properties when grown on fats. Ritchie<sup>1</sup> believes that the "fat" of the tubercle bacillus is really a wax containing a higher alcohol, and it is this wax to which the acid-resistance is due. He cites in opposition to this view: Fischer, who considers the Ziehl-Neelsen method of staining dependent upon the "greater substance wealth of the bacterial protoplasm and therefore on the greater absorptive power"; Helbing, who refers it to the presence of chitin, a constituent of the tubercle bacillus also mentioned by Bulloch and by Ruppel; Grimme, who claims it is due to a substance, not fat, in the cytoplasm; and Jolles, whose dictum is: "The character of the staining is due not to fatty substance alone, but to a peculiar disposition of protein and fat in the cytoplasm."

Camus and Pagniez<sup>2</sup> attempted to demonstrate the presence of free fatty acid in the tubercle bacillus, and to discover whether that was the cause of the acid-resistance; but while they found that the behavior of the tubercle bacillus corresponded to that of fatty acids, they confess that the method employed (treatment of the organisms with copper sub-acetate, staining with hematoxylin, decolorization with potassium ferrocyanide) is not specific for fatty acids, and further they find that not all fatty acids are acid-proof, this quality increasing with an increase in the molecular weight of the acid. Deyke<sup>3</sup> states that acid-fast bacilli owe this quality to the presence of fatty substances which are the special vehicle of the free fatty acids, the source of the acid-resistance. The neutral fats share only indirectly in the acid-fastness, being the cause of the difficulty in staining.

Auclair and Paris<sup>4</sup> oppose the view that the acid-resistance of the tubercle bacillus is a function of the fatty or waxy substance, claiming that this property belongs to all the bacillary constituents, though in differing degree. After a four months' treatment with various solvents which completely removed the fats and waxes, the bacilli were still acid-fast. The bacterial protoplasm, isolated without alteration of its chemical constitution, was found to be acid-fast.

<sup>1</sup> *Jour. Path. and Bacteriol.*, 1904, 10, p. 334.

<sup>2</sup> *La Presse méd.*, 1907, 15, p. 65.

<sup>3</sup> *München. med. Wchschr.*, 1910, 57, p. 633.

<sup>4</sup> *Arch. de méd. exper. et d'anat. path.*, 1907, 19, p. 129.

The residue, after the removal of albuminoids and fatty materials by boiling in KOH, responded to the test for cellulose and was extremely acid-resistant, even on prolonged treatment with  $\text{HNO}_3$  and absolute alcohol. Acid-fastness, therefore, they conclude, belongs to all the chief constituents of the bacillus individually, though there are "nuances" in this resistance of the different parts, the protoplasm possessing it in least measure, though still to a marked degree. The staining reaction of the bacillus itself is therefore the sum of the staining reactions of each of its constituents. These workers further consider the acid-resistance of the tubercle bacillus dependent upon both its chemical and its physical constitution. Chemically the three great groups of which it consists—fats and waxes, proteins, and celluloses—are each acid-fast. But this is not a sufficient explanation of the phenomenon. It is necessary to consider the physical state in which these constituents occur. All are greatly condensed, hence the dye penetrates slowly, usually with the assistance of heat, phenol, or anilin. Decolorizing agents are also slowly taken up, and the resistance to decolorization is the natural corollary of the resistance to staining. Fontes<sup>1</sup> states that Hammerschlag proved that in the tubercle bacillus existed a protein-like substance which was acid-resistant. Fontes himself used Auclair and Paris' method of fat extraction, testing the bacterial residue for acid-resistance after the use of each solvent. He found this residue in every instance withstood decolorization in 1:3  $\text{HNO}_3$ . Like Bulloch and Macleod,<sup>2</sup> he was able to isolate an acid-resistant wax, which was chemically one of the higher alcohols, resembling cholesterol or phytosterol. He confirmed the findings of Auclair and Paris that the bacilli remain acid-fast after treatment with fat solvents.

Gibier<sup>3</sup> discovered that bacteria grown in mixed culture with tubercle bacilli in broth acquired a certain resistance to acid; to him this fact seemed to show that the substance which retains the aniline dye in the body of the tubercle bacillus, in spite of treatment with acid, is not intra-cellular merely, but is also present in the liquid of the culture medium whence these other bacteria absorb

<sup>1</sup> *Centralbl. f. Bakteriöl.*, 1909, Orig. Abt. I, 49, p. 317.

<sup>2</sup> *Jour. Hyg.*, 1904, 4, p. 1.

<sup>3</sup> *Compt. rend. Soc. de biol.*, 1897, 49, p. 789.

it. Aronson<sup>1</sup> also believed in the existence of an extra-bacterial substance, "tubercle wax," in the form of a secretion product between the bacilli.

Very recently Benians<sup>2</sup> has carried out certain experiments on the relative resistance to acid and retention of the Gram stain of crushed and uncrushed bacilli, and the results lead him to believe that the quality of acid-resistance is a purely physical phenomenon peculiar to the intact organism. When the physical integrity of the bacillus is destroyed, as by crushing, a procedure which would in nowise alter the chemical relationships of the bacterium, he finds the resistance to acid disappears. This is a rather unique conception of the significance of the acid-fastness of the tubercle bacillus and Benians' work will be more fully discussed below.

In view of the almost universally accepted fact of the existence of a fatty or fatlike constituent of the tubercle bacillus, it seems reasonable to suppose that the bacilli might manifest a particular affinity for the so-called "fat-dyes." Such dyes have served several investigators for the demonstration of the presence of fatty substances in bacterial protoplasm as well as in the tissues of higher plants and in animals. Unna was probably the first to employ a fat-stain (osmic acid) for the demonstration of the presence of fatty material in the tubercle bacillus. Ritchie<sup>3</sup> confirmed Unna's results—finding that the cultures of the bacilli were blackened and the individuals also colored upon treatment with osmic acid. Dorset<sup>4</sup> experimented on the tubercle bacillus with sudan III. Attempts at staining the bacilli after extraction with alcohol and ether were "not satisfactory," but in pure culture he obtained "very satisfactory" results. His method was to apply a cold saturated solution of the dye in 80 per cent alcohol for five or ten minutes and then wash for the same length of time in several changes of 70 per cent alcohol, whereupon the characteristic beaded appearance of *B. tuberculosis* could be very readily noted. He gives a plate showing rods and granular forms, while the text states that "the germs are found stained a bright red, and the beaded appear-

<sup>1</sup> Ott, *Die chemische Pathologie der Tuberculose*, p. 31.

<sup>2</sup> *Jour. Path. and Bacteriol.*, 1912, 17, p. 199.

<sup>3</sup> *Jour. Path. and Bacteriol.*, 1904, 10, p. 334.

<sup>4</sup> *New York Med. Jour.*, 1899, 69, p. 148.

ance is very distinct." The stain withstood decolorization for two minutes in 4 per cent  $\text{H}_2\text{SO}_4$ ,  $\text{HNO}_3$ ,  $\text{HCl}$ , and in  $\text{NH}_4\text{OH}$  of the same strength. Dorset was further able by means of this dye to demonstrate the presence of tubercle bacilli in sputum smears, in sections of a tuberculous gland from a guinea-pig, and in sections of tuberculous lung, and gives figures of sputum smear and of lung preparation in which the bacilli are clearly visible. He decided that sudan III was a selective stain for tubercle bacilli, and proceeded to demonstrate that fact by attempting to stain various other bacteria found in sputa, as well as preparations of pure cultures of anthrax, glanders, hog cholera, typhoid, diphtheria, *Staphylococcus pyogenes aureus*, and *B. prodigiosus*, in every case with negative results. Further, he was able to distinguish, by means of this selective action of the dye for the tubercle bacillus, between it and the smegma bacillus, which also responds to the carbol-fuchsin stain; and he states that sudan III is of practical value as a stain for the recognition of tubercle bacilli where a rapid method is desired for staining organisms in tissues, and differentiating between smegma and tubercle bacilli. Ritchie,<sup>1</sup> repeating Dorset's experiments, was able by means of sudan III and scarlet R to stain cultures of tubercle bacilli, but found the individual organisms "apparently colorless." He states that "sudan III and scarlet R are dissolved by, and consequently stain, all fats, even lecithin, myelin, and lipochrome, and therefore would stain the palmitin, stearin, wax, and lecithin of the bacilli." Though these dyes serve to demonstrate the presence of fatty substance in the bacilli—without, however, indicating the specific character of this fat—Ritchie distinctly states that they do not facilitate the detection of tubercle bacilli in film preparations made from sputum, tuberculous granulation tissue, or tuberculous "pus." Nor could any stained bacilli be detected in films of a culture of *B. tuberculosis* when stained with sudan III. In two of the 13 sputa stained, and once in a tuberculous spleen from a guinea-pig, he obtained, with sudan III, red stained bodies resembling the tubercle bacillus in size and occasionally beaded; but with scarlet R his results were uniformly negative. Moreover, such success as he had with sudan III was

<sup>1</sup> *Jo ur. Path. and Bacteriol.*, 1904, 10, p. 334.



obtained by lengthening the time of staining to from one and a half to 24 hours either at room temperature or at 37° C., followed by the washing in 70 per cent alcohol, and the use of glycerol as a mount. Dahms<sup>1</sup> confirms Dorset's conclusion that sudan III is a specific stain for tubercle bacilli, smegma bacilli "invariably" failing to take the stain while "tubercle bacilli always appeared red after its application," though he naïvely adds: "While the total amount of experimental work performed with Dorset's method is perhaps still too small to warrant the assertion that the process is always trustworthy, yet further experimentation will probably prove that such is the case." LeDoux<sup>2</sup> also used the Dorset method and states that after repeated attempts he could get no results, although he has followed the directions exactly. Sputa and sections were equally negative. Fuchs<sup>3</sup> was unsuccessful in his attempts to stain pure cultures of tubercle bacilli by the sudan III method, and he considers this negative behavior of cultures and material from the animal body the more remarkable since "the demonstration of the tubercle bacilli in sputum by means of sudan III was always easily successful." Finally, Cowie<sup>4</sup> was unable, with the exact technic of Dorset, to stain the tubercle bacillus, nor did any extension of the time of exposure to the dye, even to 12 hours, produce a more favorable result. Thereupon he entered into a correspondence with Dorset, only to discover that Dorset himself had found difficulty in duplicating his earlier results, and after testing different preparations of sudan III, and learning from the manufacturers that the dye was not a pure compound and its composition varied, had been compelled to conclude that the specificity of sudan III for tubercle bacilli was a peculiarity of one particular lot of dye, and quite a matter of accident. Sata<sup>5</sup> succeeded in staining tubercle bacilli in frozen sections in sudan III, but states that the staining was very faint. Unna,<sup>6</sup> who demonstrated the presence of fatty substances in the tubercle bacillus by means of osmic acid, also investigated

<sup>1</sup> *Jour. Am. Med. Assn.*, 1900, 34, p. 1047.

<sup>2</sup> *Centralbl. f. Bakteriol.*, 1900, Orig. Abt. I, 27, p. 616.

<sup>3</sup> *Centralbl. f. Bakteriol.*, 1903, Orig. Abt. I, 33, p. 649.

<sup>4</sup> *New York Med. Jour.*, 1900, 71, p. 16.

<sup>5</sup> *Centralbl. f. allg. Path. u. path. Anat.*, 1900, 11, p. 97.

<sup>6</sup> *Centralbl. f. Bakteriol.*, 1897, Ref. Abt. I, 21, p. 938.

the staining reactions of sudan III. He states that this dye stains olein and oleic acid more readily than it does palmitic and stearic acid, and, from the staining reaction of the tubercle bacillus, infers that its fatty substance must be more nearly related to oleic acid.

J. Lorrain Smith,<sup>1</sup> in employing Nile blue sulfate for the staining of fat, discovered that it was possible to obtain a red stain by acid hydrolysis of the fat, and by boiling the aqueous solution of the dye with a few drops of  $H_2SO_4$  to secure differential staining of neutral fats and fatty acids. Fats hydrolyzed and stained resisted decolorization by 20 per cent  $HNO_3$ , a peculiarity which led him to infer that "the use of acid in the carbol-fuchsin method was probably not merely to wash out the stain from all structures save tubercle bacilli, but to hydrolyze the fat of the bacillus, and hence affect the acid-fast combination of color-base and fatty acid. Further, since this combination of color-base and fatty acid is soluble in alcohol, this might be the reason why bacilli such as the smegma bacillus are acid- but not alcohol-fast; the tubercle bacillus itself being only relatively alcohol-fast." Lastly, Eisenberg<sup>2</sup> has used a wide range of dyes variously modified, among them the common fat-dyes, for the staining of fat in bacteria. Most of the work, however, was done on anthrax, and he does not mention the staining reactions of *B. tuberculosis*.

The present work was undertaken to learn whether "fat-dyes" penetrate and stain the tubercle bacillus or its ether extract, and, having done so, withstand decolorization with acid or alcohol, in the hope that the results might throw light on the permeability of tubercle bacilli to organic compounds, and, perhaps, prove suggestive in the attempts to develop a chemotherapy of tuberculosis. The dyes used have been chiefly the sudan series (sudan III, sudan yellow, and sudan brown), scarlet R, and Nile blue sulfate; though Bismarck brown, indulin, indophenol blue, and dimethylaminoazobenzol, as well as basic fuchsin, eosin, neutral or anilin red, Janus green, methylene blue (für Bakteria and rectifiziert nach Ehrlich) and trypan blue (this last group falling quite outside the class of fat-dyes) have also been tested. The majority of the dyes have been

<sup>1</sup> *Jour. Path. and Bacteriol.*, 1907, 12, p. 1.

<sup>2</sup> *Centralbl. f. Bakteriol.*, 1908, Orig. Abt. I, 48, p. 257; *Virchow's Arch. f. path. Anat.*, 1910, 199, p. 502.

applied in the form of saturated solutions in 70 per cent alcohol, but aqueous solutions have also been employed where indicated. Smears of human tubercle bacilli from laboratory cultures, or of the dead bacilli (the residue after filtration in tuberculin preparation), which were kindly furnished us in large quantities by Dr. Paul Lewis and by Parke, Davis & Co., both as obtained and also after being washed to free them from foreign matter, constitute the mass of material stained.

All the dyes mentioned effected mass-staining of the smear after exposure for 24 hours at room or at incubator temperature. By mass-staining is meant a macroscopic staining of the smears, or of masses of tubercle bacilli on or from culture tubes. This is practically maximum exposure, smears stained for longer periods (48, 72, or 96 hours) showing no further absorption of dye. Frequently a few minutes' exposure was sufficient for mass-staining, but the fact that a mass-stain is obtained is never an infallible, and frequently no indication whatever of the absorption of the stain on the part of the individual organisms. Neither is the fat-solubility of a dye inseparably associated with its efficiency as a bacillus stain, for some effective dyes were non-fat-soluble.

The results obtained (see Table 1) are briefly as follows:

*Sudan III.*—With Dorset's technic there was no staining of the individual bacilli, nor any mass-staining in the majority of cases. Upon applying the stain hot for the same length of time, there was distinct mass-staining, but while the individual organisms could be distinguished, their color differed neither in intensity nor in tone from that of the unstained smears. Fifteen minutes at room temperature produces practically the same result. Two smears stained for that length of time in a hot solution, however, not only exhibited mass-staining, but were the only smears in which sudan III undoubtedly stained the individual bacilli. In one hour, mass-staining only occurred. As was to be expected, after 24 hours' treatment with the dye, mass-staining was the rule, nine smears showing orange-red, frequently granular masses. Two smears, however, failed to take up the dye, and in no instance were there stained individuals. Though many of the slides showed granules, these failed to exhibit the beaded arrangement characteristic of tubercle

bacilli. It was impossible by means of sudan III to detect stained bacilli in smears of "pus" or in sections of tubercle from tuberculous guinea-pigs. These results are in absolute harmony with those of Ritchie, Le Doux, and Cowie cited above, as well as with Fuchs's experience with material from the animal body. On the other hand, they are directly opposed to the experience of Dahms and to Dorset's earlier work. Sudan III, then, is useless as a stain for individual organisms. Dilute solutions of sudan III gave absolutely negative results. Most of the tests have been made with concentrated solutions of a Grübler preparation of sudan III, but an Eimer and Amend, and an Elberfeldt's Farbenfabrik preparation, called "cerise orange III," have also been tried, both with negative results. Washing in alcohol failed to remove the dye from stained preparations, but 3 per cent  $\text{HNO}_3$  caused a slight decolorization.

*Sudan yellow.*—In staining with sudan yellow, the character of the dye itself makes it even more difficult than with sudan III to determine when actual staining of the bacilli occurs. Even when unstained, the bacilli have a yellowish tinge, and the clear transparent yellow of the dye produces only a slight deepening of the hue. Slides exposed for 15 minutes at room temperature show faint mass-staining but no stained individuals, while smears exposed to hot dye for the same period were chiefly negative or doubtful, though on one, clearly stained bacilli were visible. Smears stained for 30 minutes at room temperature gave rather doubtful mass-staining and no individual stain. In one hour, there was mass-staining, and one case of undoubted staining of bacilli. The bacilli on the other smears stained doubtfully, though still much resembling unstained individuals. Twenty-four hours' exposure effects individual- as well as mass-staining. Only one smear stained for that length of time was recorded as negative, the others being doubtful or positive. This dye is alcohol-fast, while nitric acid merely intensifies the color.

*Sudan brown.*—Fifteen minutes' exposure to sudan brown, whether at room temperature or after heating the dye, gave mass-staining only. Smears treated at room temperature for 30 minutes were faintly stained *en masse*, and one showed pale brown, alcohol-

fast organisms. Smears stained for an hour, however, showed no stained individuals. In 24 hours, all smears were stained microscopically, and four showed bacilli more or less deeply stained, though on two others no trace of bacilli could be detected. In two cases, where the microscopic picture strongly suggested individual staining, this was demonstrated conclusively by treating the slide with carbol-fuchsin, whereupon a marked portion of the smear showed a distribution of fuchsin-stained organisms, corresponding to that of the brown stained bacilli. Neither 50 per cent alcohol nor 3 per cent nitric acid decolorized the stain. Because of its brownish color, it is less difficult in the case of this dye to determine whether organisms are stained or not, and in this respect sudan brown is the most satisfactory of the sudan series.

•*Scarlet R.*—Smears stained for 15 minutes in hot dye were colored *en masse*, but individual staining was decidedly doubtful, save in the case of a single smear. Thirty minutes at room temperature is also too short a time to obtain more than a mass-stain. In an hour, however, some slides showed the organisms colored a pale pink. In the greater number of smears exposed to the dye for 24 hours, the staining was negative, and doubtful in the remainder. Some exhibited dark granules, spaced as if at the ends of a bacillus. The stain was retained after treatment with alcohol and acid. While by no means satisfactory as a stain for individual organisms, or to be at all recommended for the detection of tubercle bacilli, the above results with scarlet R are rather more favorable than the findings of Ritchie (see above), which were uniformly negative; for here, in half of the smears, the bacillus-stain was doubtful or positive, though negative in the other half. In general it appeared to offer rather more possibility of use than does sudan III.

*Nile blue sulfate.*—Aqueous solutions of Nile blue sulfate stain *en masse* in 15 minutes, but even with an exposure of 24 hours the individual stain is shadowy and indefinite. In no instance did the aqueous stain cause a red colorization of the organisms, either after the smear was washed with acid or upon exposure to the air after staining, a result to be expected if, as J. Lorrain Smith claims, the staining is the combination of the dye-base with the fatty acid set free by hydrolysis of the fat, either upon washing with acid or

exposure to the action of the atmospheric carbon dioxid. Of the smears stained 15 minutes in alcoholic solution of the dye, one showed mass-staining only; the others were doubtful or showed pale blue individuals. After staining for an hour, blue, shadowy bacilli were visible, while in 24 hours, all smears exhibited doubtful individual-staining except one negative smear. Of the smears stained for 96 hours, one was doubtful, while the others showed definitely stained organisms. Neither acid nor alcohol effects decolorization.

*Janus green*.—When allowed to act for only 15 minutes or for an hour, Janus green gives only a shadowy outline of individuals. In 24 hours, the results were about evenly balanced, some smears being unstained, and others showing pale, greenish bacilli. In a single instance definitely and sharply stained bacilli of characteristic appearance were visible. A peculiarity of smears stained in Janus green is their variegated mass-staining. It was rare to find a slide that was bluish or greenish only. The majority were greenish with violet or reddish patches, but some were entirely violet or red. Alcohol does not remove the dye, but acid does, strongly, with only a short exposure.

*Neutral red*.—After one hour in the dye, individual staining is still doubtful. Mass-staining occurs, and the clumps show darker granules and a very few rods. In 24 hours, some smears still showed no stained organisms, though in others were very faintly pink bacilli. The dye withstands alcohol but not acid.

*Indulin*.—After one hour, smears show pale organisms. After 24 hours, there is undoubted individual-staining which withstands decolorization with 50 per cent alcohol and 3 per cent  $\text{HNO}_3$ . The color of the dye makes it possible readily to differentiate stained from unstained bacilli, and in this respect it is far better than the sudans.

*Indophenol blue*.—Twenty-four hours in the dye gives only a faint mass- and no individual-stain.

*Dimethylaminoazobenzol*.—In 24 hours, a faint mass-stain, far paler than with sudan yellow, was visible, with a slightly granular individual stain. Alcohol and  $\text{HNO}_3$  failed to decolorize.

*Eosin*.—Alcoholic solutions of eosin in 24 hours stain both the

TABLE I.  
ACTION OF DYE ON SEARS OF TUBERCLE BACILLI.

DYE	SOLVENT	TIME OF EXPOSURE	TEMPERATURE	TOTAL NO. OF SLIDES	MASS-STAINING		INDIVIDUAL-STAINING			DECOLORIZATION	REMARKS
					Positive	Negative	Negative	Doubtful	Positive		
Sudan III.....	70 per cent alcohol	2-3 min.	Hot	1	1	..	..	1	..	Alcohol-fast but slightly decolorized by 3 per cent HNO <sub>3</sub>	Granules
		5 min.	Cold	1	..	1	..	..	..		
		10 min.	Hot	3	1	2	3	..	..		
		15 min.	Cold	4	4	..	4	..	..		
		30 min.	Hot	2	2	..	..	1	1		
		1 hr.	Cold	1	..	..	..	1	..		
		24 hrs.	Incubator	7	7	..	6	..	..		
		48 hrs.	Cold	3	3	..	1	2	..		
		96 hrs.	Cold	2	..	..	2	..	..		
		24 hrs.	Cold	4	2	1	4	..	..		
Sudan yellow.....	(Herzheimer's) KOH	15 min.	Cold	3	3	..	..	..	..	Withstands 50 per cent alcohol, 3 per cent HNO <sub>3</sub>	Faint mass-stain granules
		30 min.	Hot	2	1	1	..	..	1		
		1 hr.	Cold	2	..	..	..	..	..		
		24 hrs.	Cold	6	6	..	1	2	3		
		15 min.	Cold	1	1	..	..	..	..		
		30 min.	Hot	2	2	..	..	..	..		
		1 hr.	Cold	3	3	..	2	1	..		
		24 hrs.	Incubator	3	3	..	2	1	..		
		24 hrs.	Cold	6	6	..	..	3	3		
		15 min.	Cold	4	4	..	..	2	1		
Sudan brown.....	70 per cent alcohol	15 min.	Hot	1	1	..	..	..	..	(50 per cent) Alcohol- and acid-fast (3 per cent HNO <sub>3</sub> )	Alcohol- and acid-fast
		30 min.	Cold	2	2	..	..	..	..		
		1 hr.	Cold	3	3	..	2	1	..		
		24 hrs.	Incubator	3	3	..	2	1	..		
		24 hrs.	Cold	6	6	..	..	3	3		
		15 min.	Hot	4	4	..	..	2	1		
		30 min.	Hot	1	1	..	..	1	1		
		1 hr.	Cold	4	4	..	1	1	2		
		24 hrs.	Incubator	4	4	..	4	..	..		
		24 hrs.	Cold	7	7	..	..	3	..		
Scarlet R.....	70 per cent alcohol	15 min.	Cold	3	3	..	..	2	..	Alcohol- and acid-fast	Alcohol- and acid-fast
		30 min.	Hot	3	3	..	..	1	1		
		1 hr.	Cold	3	3	..	..	1	1		
		24 hrs.	Incubator	3	3	..	..	1	1		
		24 hrs.	Cold	6	6	..	..	3	3		
		15 min.	Hot	4	4	..	..	2	1		
		30 min.	Hot	1	1	..	..	1	1		
		1 hr.	Cold	4	4	..	..	1	2		
		24 hrs.	Incubator	4	4	..	4	..	..		
		24 hrs.	Cold	7	7	..	..	3	..		
Nile blue sulfate....	Aq.	15 min.	Cold	3	3	..	..	2	..	Alcohol- and acid-fast	Alcohol- and acid-fast
		30 min.	Hot	3	3	..	..	1	1		
		1 hr.	Cold	3	3	..	..	1	1		
		24 hrs.	Cold	3	3	..	..	1	1		
		24 hrs.	Incubator	6	6	..	..	3	3		
		24 hrs.	Cold	2	2	..	..	2	..		
		96 hrs.	Incubator	3	3	..	..	2	..		
		96 hrs.	Cold	3	3	..	..	1	1		
		24 hrs.	Cold	3	3	..	..	2	..		
		96 hrs.	Cold	3	3	..	..	1	1		

TABLE 1.—Continued.

Janus green .....	70 per cent alcohol	15 min.	Cold	5	5	..	1	2	2	Alcohol-fast. 3 per cent HNO <sub>3</sub> decolorizes somewhat	Granules
	Aq.	1 hr.	Cold	2	2	..	2	..	..		
		24 hrs.	Incubator	3	3	..	2	..	..		
		24 hrs.	Cold	6	6	..	..	..	..		
			Incubator	1	1	..	..	..	..		
Neutral red.....	70 per cent alcohol	1 hr.	Cold	1	1	..	..	..	..	Alcohol- but not acid-fast	Granules
		24 hrs.	Cold	4	4	..	2	1	..		
			Incubator	1	1	..	..	..	..		
Indulin.....	70 per cent alcohol	1 hr.	Cold	3	3	..	1	2	..	Alcohol- and acid-fast	Better than sudan III
	Aq.	24 hrs.	Cold	3	3	..	1	..	..		
		24 hrs.	Incubator	1	1	..	1	..	..		
Indophenol blue...	70 per cent alcohol	24 hrs.	Incubator	1	1	..	1	..	..		
Dimethylamino-azobenzol.....	70 per cent alcohol	24 hrs.	Cold	1	1	..	..	..	..		Granules
Eosin .....	70 per cent alcohol	24 hrs.	Cold	1	1	..	..	1	..	Alcohol- and acid-fast	As satisfactory a stain as carbolfuchsin
	5 per cent phenol	24 hrs.	Incubator	2	2	..	..	..	2		though paler
Bismarck brown...	70 per cent alcohol	2 min.	Hot	1	1	..	..	1	1	Alcohol- and acid-fast	Never succeeded in duplicating time; 15 min. shortest time possible to get bacillus-stain
		15 min.	Cold	3	3	..	1	1	1		
		1 hr.	Cold	5	5	..	1	3	3		
		24 hrs.	Cold	6	6	..	..	3	..		
	5 per cent phenol	10 min.	Cold	1	1	..	..	1	..		
		1 hr.	Cold	1	1	..	..	..	1		
		24 hrs.	Cold	5	5	..	2	..	2		
		96 hrs.	Cold	2	2	..	..	..	..		
Basic fuchsin .....	70 per cent alcohol	15 min.	Cold	1	1	..	..	..	1	Alcohol- and acid-fast	Deep mass-stain. Rather uniform individual stain, few granules
Methylene blue <i>f. Baki</i> .....	Alcoholic	24 hrs.	Cold	1	1	..	..	..	1 (gran.)	Alcohol- and acid-fast	
	Aq.	30 min.	Cold	1	1 (heavy)	..	..	..	1 (unif'm)		
Methylene blue <i>red</i> .....	Alcoholic	24 hrs.	Cold	1	1	..	..	..	1 (faint granular)		
		30 min.	Cold	1	1	..	..	..	1 (granular)		
	Aq.	30 min.	Cold	1	1	..	..	..	1 (granular)		
		24 hrs.	Cold	1	1	..	..	..	1 (faint)		
Trypan blue.....	Aq.	30 min.	Cold	1	1	..	..	..	1 (faint)		



smears as a whole and the bacilli themselves, and the stain is alcohol- and acid-fast. Though paler, the coloration is quite as definite as with carbol-fuchsin, and, with the possible exception of basic fuchsin, this is by far the most satisfactory of the dyes tested.

*Bismarck brown.*—In the early work a smear was obtained that after two minutes' exposure to this dye showed stained bacilli, but later work has failed to duplicate this result. Fifteen minutes with heat gave pale yellow bacilli. After one hour, the results were not satisfactory, though there were shadowy organisms, and a few dark rods. In 24 hours, the individual-stain was always positive, though faint. Occasionally organisms would be beaded, but for the most part there was a uniform individual-stain. The dye is acid- and alcohol-fast.

With basic fuchsin and the two preparations of methylene blue, after an exposure of 30 minutes, as well as after 24 hours, both mass- and individual-staining were obtained, a result in accord with that of DeWitt.<sup>1</sup> It is interesting to note, in this connection, that, while treatment with the alcoholic solution of methylene blue *rect.* resulted in uniform individual-staining, the aqueous solution gave the beaded appearance so characteristic of the tubercle bacillus.

Some attempt was made to find whether mordanting a dye increased its penetrability. Herxheimer's modification of sudan III—saturated solution of the dye in alcohol, with the addition of 2 per cent KOH—was less satisfactory than the simple alcoholic solution. The mass-stain after 24 hours' exposure was exceedingly faint, while the individuals did not differ perceptibly, save perhaps by being slightly swollen, from the unstained. Acid and alcohol had no effect. Carbol-eosin stained the individual bacilli in 30 minutes, with heat, and in 24 hours, at incubator temperature, but the stain was paler than eosin alone gave, and the color was brownish instead of pink. Carbol-Bismarck-brown, however, stained the bacilli faintly in 10 minutes. In one hour, the organisms were pale yellow-brown. In 24 hours, all smears showed stained individuals and among this number was one of the few clear bacillus-stains obtained in this work. Ninety-six hours' exposure effected no improvement over 24 hours' staining. It is quite possible that very

<sup>1</sup> *Jour. Infect. Dis.*, 1913, 12, p. 68.

profitable investigation might be made in the way of modifying dyes to increase their penetrability, following the suggestions of Eisenberg,<sup>1</sup> as, for instance, mordanting the material to be stained, directly, by the use of an acid or an alkali, or indirectly, by linking these substances with the dye in solution, by the use of various solvents for the dye (formalin, glycerol, chloroform, etc.), by the addition of anilin or phenol to the dye, or by alterations of its configuration.

In addition to the staining of smears of dead tubercle bacilli on the slide, agar culture tubes of the living organism were filled with dye, left for 24 hours, and then washed with 50 per cent alcohol and smears made. The results were as follows:

Dye	No. of Slides	
Sudan III (alc.).....	2	Mass-stain, no stained individuals.
Sudan III (Herz.).....	2	" " " " " " " "
Sudan yellow.....	2	" " " " " " " " one slide shows stained individuals, the other only dry crystals.
Sudan brown.....	2	Mass-stain, pale individuals.
Scarlet R.....	2	" " " " " " " " no stained individuals.
Nile blue sulfate.....	2	" " " " " " " " granular stain of individuals.
Janus green.....	2	" " " " " " " " individuals stained.
Neutral red.....	2	" " " " " " " " well stained.
Indulin.....	2	" " " " " " " " not well stained.
Bismarck brown.....	2	" " " " " " " " rather deeper in color than with sudan brown.
Carbol-Bismarck-brown	2	" " " " " " " " yellow-brown, granular staining of individuals.

In glancing over this table there is manifest a considerable variability in the behavior of the different stains with different periods of staining, and even within the limits of the same period. There is no regularity about it. It is impossible to predict that if a dye applied for, say 15 minutes, stains the individual organisms, an hour's application will stain them more deeply, or even stain them at all; or that because one smear, stained for 24 hours, showed colored individuals, every smear stained for that length of time will do the same. To some extent this irregularity appears to be dependent upon the character of the smear itself. With dyes so uncertain in their action as most of those mentioned above—dyes which under the most favorable conditions give only pale colorings, lacking entirely the sharpness of the carbol-fuchsin stain—it is especially important that the smears be thin and

<sup>1</sup> *Centralbl. f. Bakteriöl.*, 1908, Orig. Abt. I, 48, p. 257; *Virchow's Arch. f. path. Anat.*, 1910, 199, p. 502.

uniform, and the organisms well separated. This is of twofold importance, both that the greatest opportunity be afforded for the penetration of the dye, and because, where a clump of bacilli appears colored, it is practically impossible with these dyes to say with certainty that it is the individuals which are stained rather than the mass of fatty material. This matter of the character of the smear can hardly be the entire explanation of the irregularity met with. There seem to be involved other factors, not yet identified, against which, because of their very indefiniteness, it has been impossible to guard. On the whole, these dyes seem likely to prove of very little value as stains for the tubercle bacillus, since even when there is undoubted staining of the organisms, it is not sufficiently sharp and definite to admit of the detection of the bacilli in tissue or in pus smears, especially when these are partially stained with the same dye.

A few experiments were made in staining smears of tuberculous pus from guinea-pigs, with sudan III or scarlet R, and counter-staining with methylene blue, or using Nile blue sulfate and Bismarck brown in combination. The results were absolutely negative. The primary stain produced a diffuse color which was replaced or modified by the counterstain, giving a diffuse, undifferentiated staining of the entire smear.

With the exception of Bismarck brown, and possibly of sudan brown and Nile blue sulfate, the "fat-dyes," i.e., the sudan series, scarlet R, Nile blue sulfate, dimethylaminoazobenzol, indulin, indophenol, and Bismarck brown, which is sometimes included in that category, do not stain the tubercle bacillus nearly so distinctly as do basic fuchsin, eosin, and methylene blue. Not only are these dyes insoluble or only slightly soluble in fat,<sup>1</sup> but further, they are perfectly efficient in aqueous solution. Apparently a physical affinity of the bacterial fats for the fat dyes is a negligible quantity in the staining of the tubercle bacillus.

In addition to attempts at staining the entire organism, the behavior of the ether extract of the bacilli toward the various dyes was also studied. The ether extract was prepared by Dr. H. J. Corper of this laboratory, as follows: Dead tubercle bacilli,

<sup>1</sup> DeWitt, *Jour. Infect. Dis.*, 1913, 12, p. 68.

the tuberculin residue containing glycerol, pepton, and other foreign matter, were washed in salt solution and extracted, first with a mixture of hot absolute alcohol and ether, then with hot ether. The earlier tests were made with this impure ether extract. Later, because of the discovery that it stained with almost any dye indiscriminately, this extract was ground with sand, extracted with cold absolute ether in a Soxhlet extraction apparatus, shaken out with water to remove water-soluble substances, and dried, constituting the pure ether extract. Smears of the fat were made both on the slides and on cigarette paper. It was found that great care must be taken that these smears be of uniform thickness, since frequently the mechanical difficulties of staining a thick smear gave rise to seemingly contradictory results in the action of a dye, or led to error in the comparison of one dye with another. In the earlier work, no especial attention was paid to uniformity of exposure to the dye, but it was found that all dyes stained the extract. Later, an attempt was made to establish the minimum time necessary for staining in the case of each dye, and also, by freeing the extract from proteins or other non-fatty substance whose presence might account for the ease in staining, to insure the contact of the dye with the extract of fats solely.

The results are given in Table 2. All the dyes stained the impure ether extract. In the case of the sudans and scarlet R, one second sufficed to effect staining. Neutral red and indulin required about a minute; Bismarck brown, one to five minutes; Janus green, two to ten, and Nile blue sulfate, five to fifteen minutes. Ether extract smears on the slide stained with Janus green exhibited the variegated appearance already mentioned, the colors ranging from green to violet and red. Smears stained with Nile blue sulfate, after standing for some time, occasionally showed reddish or orange patches, a result, according to Smith,<sup>1</sup> of the hydrolytic action of atmospheric CO<sub>2</sub>. Following Smith's suggestion,<sup>2</sup> the aqueous solution of Nile blue sulfate was boiled with a few drops of H<sub>2</sub>SO<sub>4</sub>, to increase the oxazone base of the dye, to whose presence is due the red coloration with neutral fats, fatty

<sup>1</sup> *Jour. Path. and Bacteriol.*, 1906, 11, p. 415.

<sup>2</sup> *Ibid.*, 1907, 12, p. 1.





TABLE 2—Continued.

DYE	TIME OF EXPOSURE	IMPURE ETHER EXTRACT				PURE ETHER EXTRACT				DECOLORIZED
		SLIDE		PAPER		SLIDE		PAPER		
		Stain	Minimum Time for Definite Coloration	Stain	Minimum Time for Definite Coloration	Stain	Minimum Time	Stain	Minimum Time	
Bismarck brown....	1 sec. 2 sec. 3 sec. 4 sec. 2 min. 4-5 min.	Faint - + + + +	5 min.	.. + faint + + + +	.. .. .. 2 min.	- - - - - +	.. .. .. 5 min. 5 min.	+ .. .. + + + +	.. .. .. 1 min.	Withstands 50 per cent alcohol and 3 per cent acid
Carbolic.....	1 sec. 1 min. 5 min.	.. .. ..	..	.. .. ..	.. .. ..	- - +	.. .. 5 min.	.. .. +	.. .. 1 min.	"
Trypan blue aq.....	30 min.	+	..	..	..	-	..	..	..	"
Eosin.....	30 min. 48 hrs.	Faint pink "	..	..	..	-	..	..	..	"
Fuchsin.....	5 min. 30 min.	+	..	+	..	+	..	..	..	"
Methylene blue rect. Alcoholic.....	30 min. 24 hrs. 30 min. 24 hrs.	+	..	+	..	-	..	..	..	"
Aq.....	30 min.	..	..	..	..	+	..	..	..	"
Methylene blue f. Baki. Alcoholic Aq.....	30 min. 21 hrs. 30 min. 24 hrs.	{ - +								

acids staining blue with the oxazine base. Smears treated with this acidified solution showed large areas stained red, while blue staining preponderated with the use of the simple aqueous and the alcoholic solution.

There was very little difference in the behavior of the pure ether extract toward the sudans and toward scarlet R. Possibly the stain in the minimum time is a little paler, but definite staining occurs in the same interval. Bismarck brown and basic fuchsin also show the same minimum for both ether extracts. In the case of the other dyes, however, a marked lengthening of the time is necessary for the absorption of the stain. Indulin requires five minutes instead of two seconds, neutral red and Nile blue sulfate, an hour, instead of one and 15 minutes, respectively; Janus green and indulin, methylene and trypan blue no longer stain. This result seems to confirm the accuracy of the inference that it was the presence of non-fatty matter in the impure ether extract that caused it to stain so readily and so universally.

In working with Nile blue sulfate and neutral red, and to a less degree in the case of Bismarck brown, it was found that smears of ether extract on cigarette paper showed definite staining only after prolonged soaking of the dye-treated smear in water. This peculiarity first came to light in comparing the effect of these dyes on smears on the slide with that of smears on paper. The former showed a coloration of the ether extract in a given time when the latter did not. Upon investigation it was found that when the smears on paper were soaked in water for from one to several hours, the dye was removed from the paper by which it had been readily absorbed, while the coloration of the smear not only became thus visible, but was also observed to be deeper than that of smears stained in the saturated dye for the same length of time, but not soaked afterward. This might be a purely physical phenomenon, the dye passing into the smear only when the absorptive power of the paper was satisfied, and water might serve this end as well as alcohol, or there might be some other explanation. Dr. DeWitt has suggested that the soaking may set free some constituent of the dye or of the extract, thereby facilitating the combination of the two, or (and this seems the more likely) that this may be a function



of dilute solutions, the soaking in water being tantamount to the dilution of the dye, which in the concentrated solution may form a precipitate over the surface of the smear and prevent its complete penetration and coloration, or, if it is a chemical process, the complete union of the dye with the fat. This theory is strengthened by the results with dilute solutions of Nile blue sulfate and neutral red. Into two portions of about 50 c.c. of water were shaken a few crystals of the dyes, so that the resulting solutions were of the same shade as the solution of one drop of the concentrated dye in 10 c.c. of water. Four smears, two on cigarette paper and two on slides, one each of the pure and the impure ether extract, were placed in each diluted dye. In 15 minutes a faint coloration was visible. The smears were left for 24 hours, when all were deeply stained, the depth of color and sharpness of outline being precisely that of smears stained a short time in concentrated dye and then soaked in water.

In connection with the behavior of neutral red, it was noted that upon first removing the smear on paper from the concentrated dye, the paper was dull red, but after soaking, the smear was deep raspberry red. A strip of the paper was stained to see how it responded to the dye, and it was found that upon a portion which had been held between the fingers there was this same difference in color from the remainder of the strip. Inasmuch as neutral red is sometimes used as an indicator for alkali and acid, and as this color was that produced by the addition of an acid to the dye, this suggested that it was an acid that caused the color change, and specifically the fatty acid of the ether extract, which, in the dilute solution, was free to combine with the dye. The behavior of fats and fatty acids toward Nile blue sulfate and neutral red was investigated, and it was found that butyric, oleic, and stearic acids readily take up these dyes from dilute aqueous solutions (one drop of concentrated dye solution to 10 c.c. of water), forming deeply colored rings beside the paler or completely decolorized solution, thus demonstrating the fact that fatty acids can remove the dye from dilute aqueous solutions. Palmitin, dissolved in chloroform, and olive oil also took up the dye, though the olive oil took up the dye, particularly Nile blue sulfate, far less readily than did the fatty acids. The result

is not entirely in accord with the findings of Smith, who was able to stain fatty acids, but not neutral fats.

In connection with the tests already described, Benians<sup>2</sup> experiments with crushed tubercle bacilli have been repeated with the fat dyes. Benians employed two methods for crushing bacteria: either grinding for half an hour in an agate mortar, or rubbing a coverslip with a grinding motion over one end of a smear after fixation upon the slide. He states that the former method is preferable, though "the results obtained by the method used for crushing fixed films is necessarily very partial and imperfect in most cases." This statement, together with those that films so crushed show under the microscope "masses of amorphous material mingled with intact bacilli" and that "the actual extent to which these bacilli are really crushed is naturally a matter of some doubt" found ample confirmation in the present instance. Although Benians' thesis, that "the bacterial substance itself does not exhibit in any marked degree the property of gram-positive staining or of acid-fastness, but both these properties are almost entirely dependent on the integrity of the cell" is directly opposed to the findings of previous workers, notably to those of Auclair and Paris, of Fontes and of Bullock and Macleod mentioned above, so far as the acid resistance of the tubercle bacillus is concerned my results are in accord with those of Benians. There is a marked loss of acid-fastness upon crushing the bacilli, as judged by the reaction toward the Ziehl-Neelsen stain and there is a change in the behavior with the fat dyes. With the exception of indulin all the dyes used in the previous work stained the uncrushed smear more deeply than the crushed, but in the case of the true fat dyes, the sudans and scarlet R, there was a further noticeable difference. Whereas the uncrushed smear showed amorphous, deeply stained masses, with here and there faintly stained objects suggesting individual bacilli, in the crushed smears, there were numerous faintly colored masses resembling clumps of undistributed bacteria, and upon these as a background were found thickly scattered, deeply staining granules, apparently once that part of the bacterial contents which caused the familiar "beaded" appearance. The occurrence of such

<sup>2</sup> *Jour. Path. and Bacteriol.*, 1912, 17, p. 199.

granules was especially marked on slides stained with scarlet R and with sudan brown. Crushing the tubercle bacillus, then, alters both its acid resistance and its behavior toward the fat dyes.

Dr. H. J. Corper has investigated in this laboratory the effects of fat dyes upon the growth of tubercle bacilli in culture media, and permits me to incorporate in this article the results of his experiments. In these growth experiments, 0.1 c.c., 0.5 c.c., and 1.0 c.c. of a 1 per cent solution of the fat dye in peanut oil were added to a 5 per cent glycerol agar. In the cases where 1 c.c. of the oil was used, the culture did not grow, the oil being present in sufficient amount to prevent growth; but with smaller amounts of oil, growth generally occurred at the border of the oil, and staining was easily observed there. The cultures were made in small bottles, rhomboid in shape, laid on one side.

The chief results of these experiments were as follows: In culture experiments masses of tubercle bacilli are stained macroscopically when in contact with oil containing sudan III, scarlet R, sudan yellow, dimethylaminoazobenzol, indulin, and Nile blue sulfate. The last two dyes displayed a marked toxicity toward the organism. Bismarck brown when suspended in oil does not appreciably stain the cultures, but when dissolved in the agar does stain the transplant. None of these dyes stains the individual bacilli. These experiments therefore corroborate and amplify the results obtained by direct staining, and show that tubercle bacilli can grow even when in immediate contact with certain of the fat dyes. It may be recalled that Dr. Corper, in a previous article of this series, reported that the fat dyes had no influence upon the course of experimental tuberculosis in animals.

#### CONCLUSIONS.

All the dyes used, whether fat-soluble or not, stain pure cultures of tubercle bacillus, *en masse*, because of the presence of stainable substances outside the bacilli.

Sudan III does not stain individual tubercle bacilli, either in smears of pure culture, in tuberculous pus, or in tuberculous tissue.

Sudan yellow and sudan brown stain the bacilli, faintly, in pure culture smears, upon prolonged exposure, or on heating.

Scarlet R resembles sudan III in behavior, but is slightly less

inefficient, about half the tests for individual staining being doubtful or even faintly positive.

Nile blue sulfate gives a faint and rather unsatisfactory bacillus-stain, as does Janus green, for the most part. A single smear stained with Janus green showed deeply stained bacilli, but this could not be duplicated.

Indulin stains the bacilli faintly, upon prolonged application.

Indophenol blue does not show any bacillus-stain.

Dimethylaminoazobenzol gives a faint and unsatisfactory bacillus-stain.

Basic fuchsin, which is only slightly fat-soluble, eosin, and methylene blue, which are not fat-soluble, stain the individual bacilli deeply in a relatively short time.

All the dyes used stained the impure ether extract of tubercle bacilli, while the purified ether extract was less readily stained by the majority of the dyes not classed as "fat-dyes."

The behavior of the dyes toward the impure ether extract corresponds with their behavior toward cultures of the bacilli, and is very different from that toward the individual bacilli. These facts seem to indicate that masses of ether-soluble substance exist on the surfaces of cultures as well as within the bacterial protoplasm, and it is with this extra-cellular material that the dyes combine.

Basic fuchsin and eosin, and to a less extent Bismarck brown, resemble the regular fat-dyes in the ease with which they stain the ether extract.

Dilute solutions of Nile blue sulfate and neutral red are more efficient than the saturated, in the staining of the ether extract.

The "fat-dyes" are not serviceable for the detection of tubercle bacilli in pus or in tissue, nor for their staining in pure cultures.

Experiments with crushed bacilli confirm Benians' view that the acid-fastness of the tubercle bacillus depends upon the physical integrity of the bacterial cell.

The fatty constituents of the tubercle bacillus are not, *per se*, the cause of the staining reaction characteristic of this organism.

This work has been done under the direction of Dr. H. G. Wells, and with the direct supervision of Dr. Lydia M. DeWitt; and to both I make most grateful acknowledgment for suggestions and assistance.

# INTRA-VITAM STAINING OF TUBERCULOUS GUINEA-PIGS WITH FAT-SOLUBLE DYES

(SUPPLEMENTARY NOTE)\*

## STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. VI.

HARRY J. CORPER.

(From the Otto S. A. Sprague Memorial Institute, and the Pathological Laboratory of the University of Chicago.)

In a preceding article of this series,<sup>1</sup> I reported the results of a series of experiments directed to determining the effects of fat-soluble dyes administered to guinea-pigs, both tuberculous and normal. Since the publication of this paper, I have completed experiments with other dyes not previously considered, and although the results are much the same, it seems desirable to report them briefly for the sake of completeness. Attention is also called to the summary of experiments performed on the effect of fat stains upon the growth of tubercle bacilli in artificial media, which is incorporated in the immediately preceding paper of Miss Sherman.

### FEEDING OF FAT STAINS TO TUBERCULOUS GUINEA-PIGS.

*Indulin (fat-soluble).*—Four guinea-pigs were inoculated with tubercle bacilli and fed a 1 per cent suspension of indulin, Grübler, in peanut oil, 2-4 c.c. every second day, during the entire period of infection, being killed on the 65th day after infection. The organs—tuberculous lymph glands, spleen, kidneys, liver, adrenals, brain, fatty tissues, and lungs—were removed, ground up with anhydrous sodium sulfate, dried, and extracted in a Greene apparatus with alcohol.<sup>2</sup> None of the organs or tuberculous areas revealed any of the blue stain except the lungs, from which the alcohol extract was a distinct blue or bluish green, and section revealed stained fat in the air passages. This was easily accounted for by aspiration of the oil, containing the dye.

\* Received for publication February 14, 1913.

<sup>1</sup> *Jour. Infect. Dis.*, 1912, 11, p. 373.

<sup>2</sup> The indulin used was soluble in alcohol and CHCl<sub>3</sub>, but practically not at all in ether, petroleum ether, and water.

*Dimethylaminoazobenzol*.—Four guinea-pigs were inoculated with tuberculosis and fed a 1 per cent solution of dimethylaminoazobenzol (Grübler) in peanut oil, 2–4 c.c. every other day, during the entire period of infection, 65 days, at the end of which time the tissues were studied microscopically (frozen sections) and chemically, as above, grinding them with anhydrous sodium sulfate and extracting with ether.<sup>1</sup> On account of the fact that most of the organs and fats of the body gave a yellow ether extract, no definite conclusions could be reached from the chemical examination, but, microscopically and macroscopically, none of the organs or tuberculous areas revealed definite yellow staining.

*Bismarck brown*.—Five guinea-pigs were inoculated with tubercle bacilli and four of these fed Bismarck brown, 1 per cent suspension, in oil, and one a 1 per cent watery solution of 2–4 c.c., every second day, the experiment lasting 65 days, at the end of which time the organs and tuberculous tissues were examined histologically and chemically by extraction<sup>2</sup> so far as possible. Most organs normally gave a brown extract with alcohol. As far as could be judged from the examination none of the organs or tuberculous areas had been stained.

Negative results were also obtained in short-period experiments of 14 days with alkanin (Fettlosl. Roth), Grübler, and Annatto (1 per cent in oil *per os*). Examinations were only histological.

*Summary*.—Indulin, dimethylaminoazobenzol, (1 per cent in oil), and Bismarck brown, (1 per cent in oil and in water), fed to tuberculous guinea-pigs, do not appear to enter the organs nor tuberculous areas to any appreciable extent when given for a period of about 65 days. Alkanin and annatto, 1 per cent in oil, do not enter within a period of 14 days.

<sup>1</sup> The dimethylaminoazobenzol used was readily soluble in ether, CHCl<sub>3</sub>, petroleum ether, and alcohol, but insoluble in water.

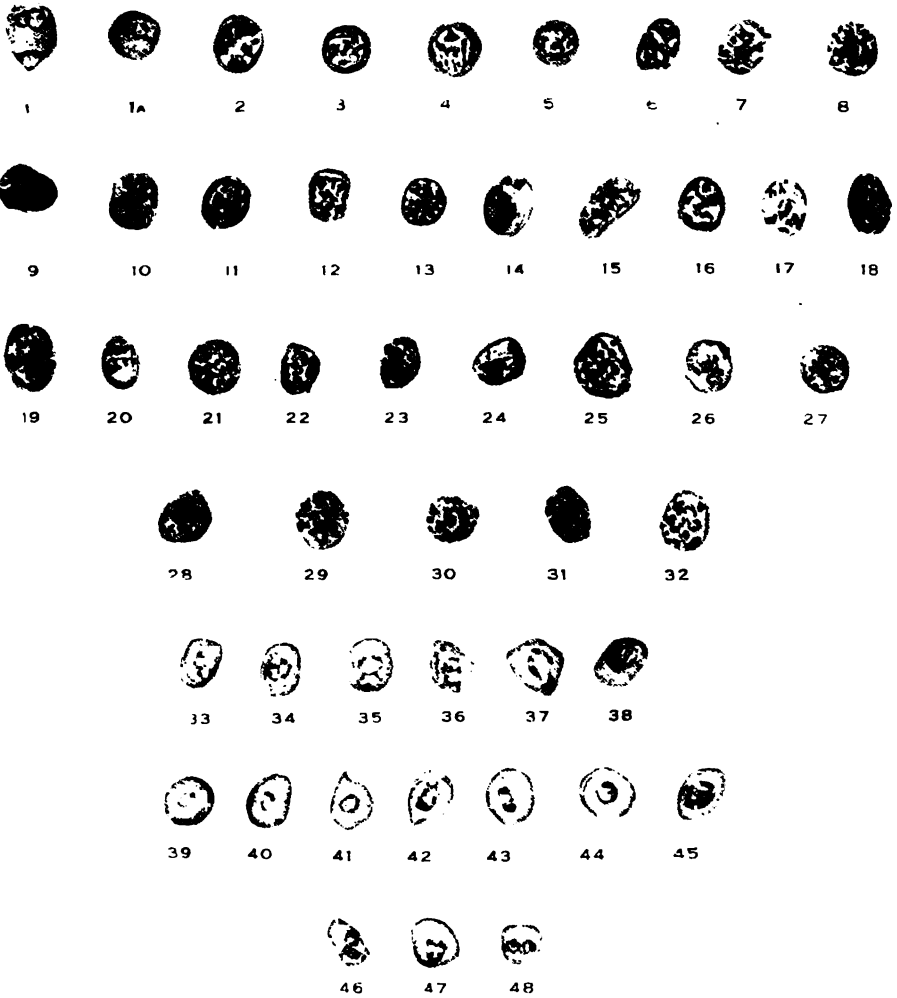
<sup>2</sup> The Bismarck brown used was soluble in water, alcohol, and petroleum ether, but insoluble in CHCl<sub>3</sub> and ether.







# PLATE I.



# *The* Journal of Infectious Diseases

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## NOTES ON THE ETIOLOGY OF RELAPSE IN MALARIAL INFECTIONS.\*

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(From the Ancon Hospital, Ancon, Canal Zone.)

(WITH PLATE I).

### I.

In a recent article Dr. Graham A. Henson<sup>†</sup> drew the attention of physicians in the southern states to the fact that while our knowledge of the transmission of malaria, the morphology of the parasites, and the methods of treatment and prophylaxis may be considered fairly complete, the important subject of the etiology of relapse is far from determined. In a general way Dr. Henson's statement is correct; but in spite of our best methods, relapses occur after all systems of quinine administration, showing that today our treatment is not seldom at fault. It may be stated also that a definite knowledge of the morphology of malarial parasites is not any too widespread, for in following closely recent literature, one often finds that descriptions of what are called "malarial parasites,"

\* Received for publication March 3, 1913.

† *South. Med. Jour.*, 1912, 5, p. 450.

or peculiar and rarely found forms of these, are such as to cast suspicion on the accuracy and correct interpretation of the observations. There are not a few who denounce many of the methods of quinine prophylaxis as worse than useless, often apparently with very good reason.

However, it is an undisputed fact that relapse in malaria is responsible for many of the evils that follow infection with one or more species of *Plasmodia*. There is some reason to believe that primary attacks, even with estivo-autumnal infections, are not as fatal as are relapses or their equivalents, repeated reinfections. In the Canal Zone often we cannot say with certainty whether any particular subsequent attack is a relapse or a reinfection; since most of us live and work in the same place, it follows that if the place can harbor the source of original infection, reinfections will surely follow if the cause is not removed.

Notwithstanding the difficulty of stating definitely the actual proportion of relapse, I do not hesitate to affirm that no inconsiderable part of the malaria here prevalent is due directly or indirectly to it. For the present by a relapse I mean "a return of the fever and parasites after the former has ceased and the latter have disappeared from the peripheral blood, reinfection being excluded. I do not include those cases in which quinine has not been administered in doses sufficient to clear the peripheral blood of parasites. Such cases are not true relapses, for the original infections are only diminished."<sup>1</sup>

To prove that a large part of malaria here (and elsewhere as well) is due to relapse, I invite attention to the following data.

1. In certain places, for example, sections of Italy and Greece, and the northern part of the southern states, where a great quantity of malarial infection is present during the summer and autumn, in the winter, when the possibility of reinfection is slight or absent, there is nevertheless a very considerable amount of the disease. This shows that when large numbers of people are infected in any community, a certain proportion, after the acute symptoms have subsided, will carry the parasites in some latent manner until a favorable opportunity supervenes for the necessary increase to produce fever. In temperate regions possibly undue exposure to cold or some other debilitating cause creates this opportunity. Here in the tropics, exposure to rain with subsequent chilling, as I shall presently show, or lowered resistance due to other disease, or insufficient food, very likely act similarly. These statements are qualified because

<sup>1</sup> *Proc. Canal Zone Med. Soc.*, 1910, 3, p. 29.

until the entire etiology of relapse is definitely proved, I do not care to set forth as fact what depends on circumstantial evidence, however well founded.

2. It is not uncommon to witness a relapse of malaria, in the wards of this hospital, during the course of another disease, but that it occurs more often after convalescence is well established. If, as in the latter instance, a relapse takes place while the patient is resting, surely it is not unreasonable to suppose that relapses occur in greater numbers among those whose resistance is lowered by exposure and hard labor.

3. In the Canal Zone, it has been shown elsewhere<sup>1</sup> that the increase in the amount of malaria at the beginning of the wet season is out of proportion to the increase in the number of Anopheles mosquitoes. It takes time for mosquitoes to breed, to become infected themselves, and so to transmit infection; but the malaria rate rises rapidly and promptly immediately after the first heavy rains, even in Zone towns in which the mosquito rate shows no perceptible increase. Although the rains may drive the Anopheles indoors, thus adding to chances of infection or reinfection, still this increased number of mosquitoes indoors is not sufficient to account for the increased malaria rate. Further, at the close of the wet season, the malaria rate falls promptly with the cessation of the rains, and much more rapidly than does the mosquito rate. These facts show a very circumstantial relation between exposure to wet and chilling and the increase in the malaria rate, and between the cessation of this exposure and the decrease in the malaria rate.

4. Insufficiently treated infection with any species of *Plasmodia* is exceedingly prone to relapse, however apparent the temporary cure may have been. In the opinion of those whose experience enables them to speak with authority, quartan infection, though well treated, relapses more frequently than does infection with estivo-autumnal or tertian parasites. As far as my limited experience with quartan infection goes, the opinion is correct.<sup>2</sup> But the number of quartan infections in the Canal Zone is too small to permit of accurate deductions. Still, it is a matter of common knowledge here that estivo-autumnal infections are as a rule much more difficult to eradicate, and require much more extended treatment to effect a cure than tertian infections, although some tertian infections are very resistant to treatment. I have recently seen a patient who had five relapses with tertian malaria in three months, the last after an intravenous injection of 22.5 grains of quinine, and quinine by mouth for 10 days, in doses of 10 grains three times a day.

As a rule, owing to local conditions, estivo-autumnal and tertian infections among the Negroes and European laborers receive the same treatment and for practically the same time, the average stay in hospital being about a day longer for estivo-autumnal infections. If primary infection or reinfection were responsible for most of our malaria at the beginning of the wet season, tertian malaria should increase proportionately with estivo-autumnal, because Dr. S. T. Darling<sup>3</sup> has demonstrated that *A. albimanus*, the most common malaria carrier here, is as susceptible to infection with the one species as with the other. It has also been shown that while the gametes of *P. falciparum* (the estivo-autumnal parasite) persist longer in the peripheral circulation than do those of *P. vivax* (the tertian parasite), the latter occur here relatively more often. I find gametes in nearly all tertian infections, and crescents in amounts sufficient to infect mosquitoes in a small proportion only of estivo-autumnal infections. If the increase in the malaria rate at the beginning of the wet season were due

<sup>1</sup> Deeks, W. E., and James, W. M., *A Report on Hemoglobinuric Fever in the Canal Zone*, 1911, p. 19.

<sup>2</sup> *Proc. Canal Zone Med. Soc.*, 1910, 3, p. 29.

<sup>3</sup> *Ann. Trop. Med. and Parasit.*, 1910, 4, p. 179.

solely to primary or subsequent infections, these facts show that tertian malaria should, as has been noted, increase proportionately with estivo-autumnal. But the contrary is true, for in European laborers, who are very susceptible to malaria, and in Negroes, who are comparatively immune, the estivo-autumnal rate at this time rises, relatively as well as proportionately, greatly when compared with the tertian rate. Particularly is this true among the European laborers.<sup>1</sup>

Without relapses, then, the malaria rate should rise synchronously with the increase in the mosquito rate; but with relapses, if these produce gametes in the same proportion as primary infections or reinfections, as much evidence seems to indicate, the malaria rate is augmented not only by the number of cases due to relapse, but by the increase later in primary and subsequent infections due to the greater quantity of infection in mosquitoes, though the total number of mosquitoes may remain the same for some time.

These data will, I trust, convince of the importance of relapse as a factor in the prevalence of malaria here and elsewhere. Relapses are of importance to the sanitarian because, as Dr. Henson points out, if all primary cases were cured so that gametes could no longer be produced, and relapses with gamete production were prevented, malaria would thereby be eradicated. Relapses are of importance to the physician, since what is called "chronic malaria" results as much, if not more, from them as from reinfection. Further, a relapse means insufficient treatment of the primary infection, and those who are content to effect a temporary cure without regard to future evil would do well to remember this. One might as well treat syphilis with one or two doses of mercury, as to treat a malarial infection with five grains of quinine three times a day for a week and expect a cure.

## II.

In September, 1911, the Department of Sanitation of the Isthmian Canal Commission sent out to various authorities on malaria throughout the world a circular letter, in which information was requested as to the amount of malaria due to relapse, the various predisposing causes, the prophylaxis of relapse, and the etiology and subsequent effects. The purpose was to obtain data upon which a research could be made with the object of excluding or verifying various hypotheses current at the present time as to the etiology of relapse, and the best methods of prophylaxis and treat-

<sup>1</sup> Deeks, W. E., and James, W. M., *A Report on Hemoglobinuric Fever in the Canal Zone*, 1911, p. 26.

ment against it. Nearly all replied to these letters, and the information thus collated is now being used in an effort to obtain definite conclusions. As it is impossible at this time to present a full account of the work done in the past year, I shall limit myself in this paper to a discussion of the various hypotheses as to the etiology of relapse, and the results of my work during the last 12 months in studying anew this question in the light of the data which has so generously been furnished me.

Until the true reason for the cause of relapse is known, the treatment of malaria will remain somewhat empirical, for practically all of the authorities referred to above agree that a very large proportion of malaria, from 50 per cent to 90 per cent in malarious communities, is due to relapse; and further, as stated in the introduction, such relapse is very largely responsible for the continued propagation of malaria. Though in varying proportion, relapses have followed every method of giving quinine, from prompt and vigorous treatment of the original attack by large doses of quinine administered intravenously, to small doses in quantity just sufficient to control the fever, and no more. This means either that quinine as exhibited today is not administered properly, or that the drug is not the specific that most textbooks hold it to be, in so far as it effects a permanent cure. I do not mean that quinine is a failure, or that I would advocate the use of another drug. I wish only to emphasize the fact that as long as relapses are responsible for the present proportion of attacks of malaria, the proper treatment has not been effected. Undoubtedly the fault is by no means altogether with the drug; perhaps the perfect method of administration has not yet been found. When it is, there will be no more malaria in properly treated communities, as Dr. Henson has noted. In the meantime, the determination of the true cause of relapse is a matter of highest importance, for it is beyond all question that this cause is intimately concerned with some stage in the life-cycle of the *Plasmodia* over which quinine has little or no control.

It is my purpose at present to advance certain propositions as to some factors common to relapse, that are derived from the extended clinical observations and experience of the authorities

referred to above; and to apply the data deduced from these latter to the various hypotheses that have been advanced as to the etiology of relapse. I shall add, also, the opinions which I hold at present as a result of my work on this subject.

1. Today, as in the past, relapse is a very common feature of malarial infection.
2. Relapse almost invariably follows the so-called spontaneous cure of primary cases of malaria; that is, the cessation of symptoms without treatment.
3. Infections treated insufficiently with small doses of quinine will in all probability relapse.
4. The sooner prompt and vigorous treatment is instituted after the onset of the primary attack, the less likely is relapse to follow; and conversely, the later the primary attack is treated, even with large doses of quinine and for a long time, the more certainly will the symptoms recur.
5. Sometimes a relapse, with parasites in the peripheral blood, will take place although the patient has not stopped taking quinine. These cases are rare, but I have noted several in Ancon Hospital.
6. If reinfection is excluded, and death does not take place during a relapse, in time the infection will die out. Otherwise there would be no immunity, and every untreated case would end in death. This applies in the last analysis even to persons who are "carriers," and who manifest no febrile symptoms, although parasites may be found in the peripheral blood.
7. It is easier to effect a permanent cure for persons in good health prior to the attack, than for those in whom the malarial infection is complicated by bodily weakness or intercurrent disease, especially syphilis.

The hypotheses as to the cause of relapse fall under two heads:

1. Some particular, or peculiar, form of the parasite is developed during the asexual cycle, and this form is resistant to quinine and to the protective forces of the body. It remains latent until called into activity by certain forces whose action is not as yet clearly understood, when it breaks up into merozoite-like parasites, which take up anew the asexual cycle.

This is the hypothesis which explains relapses either as due to parthenogenesis of the female gamete, or macrogamete, as advanced by Cannalis,<sup>1</sup> Grassi,<sup>2</sup> Maurer,<sup>3</sup> Neeb,<sup>4</sup> and others, and worked out in its greatest detail by Schaudinn;<sup>5</sup> or as due to segmentation of a parasite formed by conjugation of two young asexual forms, which is the view taken by Craig.<sup>6</sup> Mannaberg<sup>7</sup> believes that

<sup>1</sup> Cited by Deaderick, and by Marchiafava and Bignami.

<sup>2</sup> *Ibid.*

<sup>3</sup> *Centralbl. f. Bakteriol., Orig.*, 1902, 32, p. 695.

<sup>4</sup> *Arch. a. d. k. Gesundheitsamte*, 1903, 19, p. 169.

<sup>5</sup> *Jour. Trop. Med.*, 1910, 13, p. 98.

<sup>6</sup> *Jour. Infect. Dis.*, 1910, 7, p. 285.

<sup>7</sup> "Malarial Diseases," Nothnagel's *Encyclopedia of Practical Medicine*, Philadelphia, 1905, p. 52.

crescents are formed by the union of two or more of these young forms, but I can find no statement of his which would indicate that he upholds the parthenogenetic hypothesis;<sup>1</sup> while Ewing,<sup>2</sup> who also observed conjugation, states: "The large size of the sporulating forms developed from conjugating pairs suggests that this process is intended to especially favor the multiplication of the species in the human host." Ewing's observations were made, as were those of Schaudinn, on the tertian parasite, while those of Craig were made on all three species.<sup>3</sup>

2. After a diminution in their vitality, due either to quinine or to the natural protective forces of the body, the asexual parasites continue to go through their normal life-cycle, but in very small numbers (relatively speaking), until the causes that bring about relapse effect a renewal of vitality, so that the parasites increase in number until the clinical symptoms of relapse are manifested.

This hypothesis has the support of Ross and Thomson,<sup>4</sup> and, slightly modified, of Bignami<sup>5</sup> also. The last believes that the parasites in their asexual cycle may become immune, either to the natural protective forces of the body, or to the action of quinine.

These hypotheses have two factors in common: the renewal of the asexual cycle to a degree when clinical symptoms are manifested; and a resistance of the latent parasites, whatever may be their form, to quinine.

In discussing these hypotheses I shall refer only briefly to the *a priori* reasons in favor of them, or to the contrary. These are discussed at length by Ross, Craig, Deaderick,<sup>6</sup> Bignami, and Henson. It is my purpose now to examine these hypotheses in the light of the propositions above mentioned, and to bring out certain facts derived from my own observations and those of others.

<sup>1</sup> Ross, in the *Prevention of Malaria* (p. 110), quotes Mannaberg as stating, in 1894, that relapses in localities free from malaria are probably due to "crescentic bodies."

<sup>2</sup> *Clinical Pathology of the Blood*, New York, 1903, p. 454.

<sup>3</sup> Craig states that Ewing also observed conjugation in the estivo-autumnal species.

<sup>4</sup> *Ann. Trop. Med. and Parasit.*, 1910, 4, p. 137.

<sup>5</sup> *Sulla Patogenesi delle Recidive nelle Febbri Malariche, Estratti degli Atti per gli Studi della Malaria*, Rome, 1910, Vol. 11. Translated in *South. Med. Jour.*, 1913, 6, p. 79.

<sup>6</sup> *Practical Study of Malaria*, Philadelphia, 1910, p. 124. Also "Recurrences in Malaria, Their Cause and Prevention," *Bull. Soc. Path. Exot. Par.*, 1910, 3, p. 498.



## THE FIRST HYPOTHESIS.

Although observers had claimed parthenogenesis as a cause of relapse prior to Schaudinn's publication, he was the first to see the process in a species of malarial parasite other than the estivo-autumnal, and to describe it minutely. In the examination of the blood of a patient who had at the time a relapse of tertian malaria, he found certain changes in the macrogametes which he interpreted as parthenogenesis, or reproduction by the female parasite without fertilization. Blüml and Merz,<sup>1</sup> and Karrewij,<sup>2</sup> later reported findings in tertian infections similar to those of Schaudinn, and Neeb, and Maurer, described sporulation of the female crescent. Later Major W. S. Harrison<sup>3</sup> published an account of parasites found by him in a case of tertian infection, which seemed to correspond with those described by Schaudinn as parthenogenetic forms, and gave very excellent illustrations. Major Harrison was reserved in his interpretation of these forms, which he found in two cases, one of relapsing malaria, and the other very probably from a relapsing case. As far as I am aware, no confirmation of parthenogenesis has been obtained accurately in quartan malaria, notwithstanding the well known tendency of this type to relapse.

The essential feature in all descriptions of the process, including and following that of Schaudinn, is the division of the chromatin into two parts. One of these, the *Restkörper*, degenerates, or at least has nothing further to do with segmentation, while the other undergoes amitotic division and forms the nuclei of merozoite forms, as in asexual division.

Craig claims that in all three forms of malarial infection there is, when conditions are favorable, a conjugation of two young asexual organisms within the same erythrocyte, with subsequent union of nuclei and cytoplasm. From this union results a parasite which grows rapidly, differs in appearance from the organisms of the sexual and asexual generations, and is resistant to quinine. Craig has not followed these organisms from conjugation to complete sporulation but has witnessed all stages, including the actual

<sup>1</sup> Cited by Deaderick, *Arch. f. Schiffs- u. Trop. Hyg.*, 1908, 12, p. 249, and by Bignami, from *Geneeskundig Tijdschrift voor Nederlandsch-Indië*, 1908.

<sup>2</sup> Cited by Bignami and by Neeb.

<sup>3</sup> *Jour. Roy. Army. Med. Corps. Lond.*, 1909, 13, p. 647.

conjugation of the living organisms, to the full grown forms, which latter he interprets as the latent organisms. Craig<sup>1</sup> states:

"These developing parasites are distinguished from other forms of the plasmodia by the collection of the chromatin in two or more irregular deeply staining masses distributed about the center of the organisms. In some instances the chromatin masses are numerous and are collected at one side of the organism, giving rise to an appearance suggesting sporulation. Such forms are very suggestive of the parthenogenetic organisms pictured by Schaudinn. [Craig notes that his older forms are very rare in the peripheral blood.] . . . They are certainly not macrogametes undergoing parthenogenesis, as they occur only when conjugation is present, and very frequently before gametes have appeared in the blood. In estivo-autumnal infections these large pigmented forms are only observed in the blood obtained by splenic puncture, and they are not observed in any form of malarial infection in which intracorporeal conjugation is absent."

Craig adds as his belief that some of these bodies are the same as those considered by Schaudinn to be macrogametes undergoing parthenogenesis; and Deaderick, who upholds the parthenogenetic hypothesis, states that while the forms observed by Craig may result from conjugation, "there is as yet no positive evidence that the latter are not macrogametes," a partial acceptance of Manna-berg's hypothesis as to the formation of gametes.

From the above statements it is very likely that some of the same forms of parasite have been observed both by Craig and by Schaudinn; the correct interpretation of their origin, function, and ultimate fate is the point in doubt.

The points in favor of Schaudinn's hypothesis may be summarized as follows:

1. The persistence of the gametes in the peripheral blood after the disappearance of the asexual parasites. Especially in tertian and quartan infections do the macrogametes persist. It is the consensus of opinion, and my own observations tend to confirm this; that in estivo-autumnal infections both microgametocytes and macrogametes persist. Since gametes of all species are believed to be notoriously resistant to quinine, it is thought probable that they alone survive to renew the asexual cycle.

2. Schaudinn's hypothesis also partly fits the propositions that I have previously advanced, because:

- a) Gametes are common features of untreated, improperly treated, and protracted malarial infections.

- b) They occur most frequently in these.

- c) They do not, however, occur at the onset of the clinical symptoms in primary attacks. But in tertian infections they occur earlier than in estivo-autumnal and quartan primary attacks. And, as noted, it is the promptly treated primary infections that are less likely to relapse.

<sup>1</sup>*Op. cit.*, p. 309.

None the less, despite this favorable evidence, I do not think that further observations, and the opinion of those who have studied the blood in many infections and over a period of some years, will justify an acceptance of Schaudinn's views. Bignami objects to Schaudinn's hypothesis on two points: he does not believe that the observations of those who claim to have confirmed Schaudinn's hypothesis were well founded, and he criticizes the work of Schaudinn himself as follows:

"But it is a fact that only once, and in one patient only, at the beginning of a relapse, the whole series of forms occurred. In spite of this he [Schaudinn], after he had given a most accurate account in his report, expressed the *conviction* that relapses after long intervals owe their origin to macrogametes, which live a long time, and have the capacity, according to him, of undergoing a reversion to the form of schizonts.

"Although the work of Schaudinn was put out in 1903,<sup>1</sup> the process that he described has not received even the most infrequent confirmation by new observations.<sup>2</sup> The unconfirmed opinion based on that report has been listened to with favor by some, as though it were a datum of fact at this time unassailable."<sup>3</sup>

Further, Bignami cites evidence to show that the supposed confirmation of Schaudinn's work rests upon unconnected and isolated observations. In most cases when the supposed parthenogenesis occurred, sporulation or other asexual forms were also observed either at the same time, or shortly before. This is true, even of Schaudinn's observation, as a careful reading of his paper in the light of subsequent knowledge will demonstrate that these were by no means excluded.

I believe, as a result of my observations in the past few years, that Bignami's objections are very well founded. It is well known that when the environment is unfavorable, any species of protozoon will take on atypical developmental and reproductive forms. Craig<sup>4</sup> has accurately described the changes due to quinine in the malarial parasites, and I have noted that the same changes are to be seen in the organisms in untreated infections, when the parasites are lessening in number. Particularly is this true of the segmenting and presegmenting forms. In these, very often the entire chromatin is not used as nuclei for the succeeding generation, but sometimes as much as half of it remains undivided. Such parasites,

<sup>1</sup> 1903 is the correct date.

<sup>2</sup> That is, not that the forms as described do not occur, but there is no evidence to prove that they are responsible for relapse.—W.M.J.

<sup>3</sup> *Op. cit.*

<sup>4</sup> *Loc. cit.*

however, do not attain the size pictured by Schaudinn for his parthenogenetic forms, which fill the entire swollen erythrocyte. But in anemic blood, such as is found in persons who are suffering from relapse (when the infection is heavy so that many forms may be observed), the environment is also unfavorable for the development of a typical life-cycle, because the erythrocytes themselves do not contain enough nourishment for the rapidly developing parasites. It is well known that the parasites depend on the hemoglobin content of the erythrocytes, which they transform to pigment, for food. The occurrence of these very heavy infections in decidedly anemic persons has not been common in my experience (relatively speaking), but I have witnessed such infections several times, and with all three species of parasites. In the blood of these patients are found very large parasites, which show marked vagaries in development, especially in tertian and quartan infections. In estivo-autumnal infections, the changes are slight, if any. These forms correspond in every way to those pictured by Schaudinn, but I cannot interpret them as sporulating macrogametes, because every phase of transition between these and normal segmenting parasites may be observed, whereas the macrogametes very early take on and keep until maturity a distinct developmental cycle. Moreover, my slides have been examined by Dr. Fülleborn, of the Hamburg School of Tropical Medicine, and he informed me that the forms just described are identical with those described by Schaudinn for parthenogenetic forms of the macrogamete. Dr. Fülleborn had seen Schaudinn's original slides, and had observed similar forms of parasites in other specimens. He was also of the opinion that the forms in question were probably atypical developing parasites. In a quartan infection that had persisted untreated for six weeks, in which the anemia was profound, as to number and hemoglobin content of the erythrocytes, I observed parasites of extraordinary shape and size, some 10 m. in diameter, and with most remarkable phases of chromatin division in the nuclei of the adult parasites. But between these and normal quartan parasites was every transitional phase.

It may be objected that if there is not sufficient nutriment the parasites could not attain so large a size. But Calkins<sup>1</sup> has shown

<sup>1</sup> *Protozoology*, New York, 1909, p. 102.

for *Paramecium*, in unfavorable circumstances, that while many of the forms were stunted, as was also the case in the malarial infections to which I have just referred, some organisms, in which division was not complete by reason of lack of vitality to finish it, attained a size out of all proportion to the normal cycle. I have observed a similar phenomenon in *Entamoebae* in which two nuclei were plainly visible without division of the cytoplasm. It does not follow that the cytoplasm loses its power of growth simultaneously with that of the nuclear material. As I have stated, such parasites are not often found in malarial infections, and perhaps because of this rarity, they are interpreted as explaining obscure phases of the life-cycle. The same mistake has been made, and very likely will continue to be made, in the interpretation of seldom found forms in other pathogenic protozoa.

It has been noted that Schaudinn's hypothesis has not received any definite confirmation. With the exception of Major Harrison's report, referred to above, I can find no writers, other than those quoted by Deaderick and Bignami, who claim to have witnessed the process. Deaderick<sup>1</sup> says:

"Golgi plainly stated it as his belief that the crescent was the parasite of fevers recurring at long intervals.

"Canalis, in 1889, described and pictured spherical bodies derived from crescents in the act of sporulation. In 1890, Antoseli and Angelini confirmed the observation of Canalis. Lewkowicz reported, in 1897, that he had seen sporulating crescents some of which contained as many as 30 spores.

"Grassi expressed the opinion in 1901 that the parasites of malaria underwent a parthenogenetic cycle whereby the species was perpetuated after the death of the schizonts.

"Maurer, in 1902, observed a sporulation of estivo-autumnal gametes, and construed it as parthenogenesis.

"Ziemann believes that he has seen parthenogenetic reproduction of quartan gametes.

"Blüml and Metz observed sporulating parthenogametes in six preparations taken from five patients with tertian malaria. The process was identical with that described by Schaudinn. Young and sporulating schizonts and young gametes were present in these same preparations."

In addition, are the observations of Karrewij, in tertian malaria, cited by Bignami, and of Neeb, which latter I have examined.

Much of this literature is inaccessible to me, but I can cite

<sup>1</sup> *Op. cit.*

opinions of competent authorities who have given it careful consideration.

Marchiafava and Bignami state:<sup>1</sup>

"Grassi and Feletti claim to have seen two forms of segmentation of the crescents, one of fission scarcely begun, the other of completed fission, similar to what is seen in the parasites of the regular fevers. Canalis describes the sporulation of round bodies of semilunar origin, and even gives a drawing of it. Golgi held that in the crescents there was a 'process of internal differentiation,' which led to the emission of young parasites which invade new red corpuscles, whence occur renewed febrile attacks. . . .

"Those who have held that there was a multiplication of the crescent bodies without being able to demonstrate it, based their belief upon the fact that the crescents persist in the blood during the apyretic interval separating a group of febrile paroxysms from the relapse, and thought that the latter could be explained only by a process of sporulation of the crescents themselves. Those who have described and pictured this sporulation were evidently led into error by their preconceived notion, and mistook a degenerative process of disintegration for sporulation; this was the case with Canalis, for instance, who, as Bignami notes, described a sporulation in which, to judge from his own designs, the nucleus took no part at all.

"We ourselves, basing our belief upon numerous and careful observations, have always held that the crescents do not multiply in human blood. This affirmation of ours, of which recent experimentation has shown the truth, was based (1) upon the fact that even under the best conditions of research we never succeeded in finding a fission form of crescents, which could with certainty be held to be a sporulation; and (2) upon the fact demonstrated by the researches of Bignami and Bastianelli, that the relapses of the fever are not in relation to the development of the crescent bodies."<sup>2</sup>

It should be carefully noted, also, that designs of malarial parasites, and interpretations of these, based upon the imperfect methods of staining before the introduction of the Romanowsky method and its modifications, cannot be accepted unless confirmed by this method. For this reason alone the observations of Canalis and those who published confirmatory reports which they have not verified by subsequent research cannot be accepted.<sup>3</sup>

Of more interest are the comparatively recent reports of Maurer and Neeb, who also claim to have witnessed parthenogenesis of the female crescent. As the reports of these observers are readily accessible, I shall not go into any detail in describing their claims.

<sup>1</sup> *Malaria, Twentieth Century Practice*, New York, 1901, 19, p. 48.

<sup>2</sup> This criticism, in part, applies also to various observations on the parthenogenesis of tertian macrogametes.

<sup>3</sup> For instance, it was long believed that there was a stage in the cycle of the tertian and quartan parasites in which the nucleus could not be demonstrated, because it did not stain by the methods then in use.

Maurer gives two drawings only, which show this so-called segmentation. He himself is not certain of these, for in the description of one of them he places a question mark after the word "gamete." His "Teilungsfigur eines Gameten(?)" cannot in any way, in so far as the picture is a criterion, be differentiated from ordinary asexual segmentation, while the representation of beginning segmentation in the *male gamete* ("männlicher(?) Gamet") will certainly not convince the experienced observer that such was the case.

Neeb found the forms which he describes in one case only, "after two and a half years of fruitless research." Before publishing his report, he sought the advice of the experienced authorities of the German and French Schools of Tropical Medicine. Neeb, with a fairness somewhat rare among those who have published accounts of parthenogenesis, states in detail the objections against his interpretation. As I shall presently give Bignami's criticism of Neeb's views, I will state here only my own opinion. Two of his figures resemble presegmentation, though somewhat atypical, of asexual parasites, as much as they resemble parthenogenesis. The third resembles in some respects a sporulating crescent, and in others beginning reduction, such as is sometimes met with if the slides are not immediately dried. Unfortunately, the plate is in black and white, and not colored. Moreover, a careful comparison of Neeb's drawings with Maurer's demonstrates that the representation by the latter of various phases of the adult asexual form is almost identical with the former's pictures and description. (Compare Maurer's figures 17, 18, and 19 with Neeb's plate.)

Bignami, in a lengthy and minute analysis of the claims of Schaudinn, Karrewij, and Merz and Blüml for parthenogenesis of the tertian gamete, and of Neeb for that of the female crescent, concludes that even if parthenogenesis did take place, which he doubts, the fact by no means implies that it had anything to do with the genesis of relapse. Bignami points out that in the first place Karrewij saw, in one case only, forms similar to those described by Schaudinn. In the second place the patient already had had several attacks of fever, when that one occurred

unexpectedly, at the beginning of which Karrewij observed parthenogenetic forms. Bignami<sup>1</sup> states:

"Now, it is evident that, to explain the cause of relapse, we should expect to find the said forms at the beginning of the first attack of the relapse itself, while everything tends to the belief that the following febrile attacks in the relapse are produced, as a rule, by the regular multiplication of the forms pertaining to the pyrogenous cycle. Therefore the observation of Karrewij is not important concerning the origin of relapse, in the strict sense of the word, but would also lead to the belief that in the ordinary sequence of attacks the gametes might reproduce themselves either entirely, or in company with other forms of the parasites."

Again, according to Bignami, Merz and Blüml found not only the supposed parthenogenetic reproduction, but at the same time common asexual sporulation.<sup>2</sup> These authors observed very heavy infections, in which the sporulating asexual forms were more than usually abundant. Moreover, in two of these cases there was doubt, as the authors admitted, as to whether they were dealing with relapse or reinfection. Bignami<sup>3</sup> objects to the interpretation of parthenogenesis in these cases:

"Now, to diagnose macrogametes in division, and to distinguish such from the numerous schizonts that were found in division at the same time in the preparations, the authors (Merz and Blüml) relied on the description given by Schaudinn and admitted, without further proof, that the distinctive characters of macrogametes in division had been previously ascertained and definitely postulated."

Concerning Neeb's views, Bignami<sup>4</sup> says:

"The observations of H. M. Neeb are even less conclusive than those that I have mentioned. He refers in his work to having seen in tertian malaria some forms similar to those described by Schaudinn, and adds drawings and a description of three forms of parasites which he interprets as crescents in parthenogenesis. He found these in two preparations from a patient ill with tropical fever, but does not state how long the fever had lasted. It is true, however, that the opinions expressed by various observers who examined Neeb's specimens were not in agreement as to two of the forms; although Prowazek interpreted them as parthenogenetic forms, Le Dantec showed the similarity of these same forms to the ordinary schizogony of estivo-autumnal parasites, and was reserved in his judgment. . . .

"As for the third form in question, Neeb himself admits that he cannot give a precise opinion."

I am of the opinion that a careful evaluation of the above data will not permit of a statement that parthenogenesis is an established phenomenon in the life-cycle of the malarial parasite, or

<sup>1</sup> *Op. cit.*

<sup>2</sup> Deaderick states this also.

<sup>3</sup> *Op. cit.*

<sup>4</sup> *Op. cit.*



that it is above all the explanation of the etiology of relapse. As Bignami notes, the confirmation is entirely unsatisfactory, and is based on unconnected and isolated observations. To which I venture to add that relapse is one of the common phenomena of malarial infection, and that the cause, when found, will be associated with very many malarial infections, and not with uncommon and seldom observed features of the life-cycle of the parasites.

There are, however, greater objections to attributing the cause of relapse to parthenogenesis of macrogametes than those I have cited. While there may be morphological grounds for the correctness of Schaudinn's hypothesis, biologically the hypothesis is at fault. It is commonly believed today that the gametes are individual parasites endowed *per se* with extraordinary power of resistance against quinine and the protective forces of the body, so that they persist in greater or less number for a considerable time. But the recent work of Thomson<sup>1</sup> in the Liverpool School of Tropical Medicine has demonstrated the error of this belief. The gametes, as individuals, do not persist, but it is an asexual cycle, which produces the gametes, that persists. Thomson showed that gamete formation depends on a function of the asexual generation, and he demonstrated very plainly that the individual gametes do not live very long in the peripheral circulation. So that when gametes persist day after day, in constant number, it is certain that an asexual cycle is simultaneously pursuing its development also, perhaps in the internal circulation.

Ross and Thomson,<sup>2</sup> moreover, have shown that such a cycle does renew its vitality, if not properly treated, without any visible necessity of parthenogenesis.

Also, it has been possible for me to demonstrate in a series of slides from autopsy cases that, in untreated malaria, all ages of crescent formation may be present, but in proportion to the amount of quinine given and the time in which it has been exhibited, young crescents were found in lesser ratio to adults, until after a week or so; provided there was no organic or other disease to interfere with proper absorption of the drug, only adult crescents, and no

<sup>1</sup> *Ann. Trop. Med. and Parasit.*, 1911, 5, p. 57.

<sup>2</sup> *Ibid.*

asexual forms were found. Young crescents were always associated in these slides with an asexual generation, showing that the production of the former is a function of the latter. Together, the work of Thomson on the crescents in the peripheral blood, and that of mine on their relation to the asexual cycle at autopsy, show that when the asexual generation is killed, the persistence of crescents stops within two weeks, generally in 10 days. This indicates, as Thomson has pointed out, that the life of the individual crescent is about 10 days, and if these organisms persist after that time, it is because the asexual cycle has not been killed.

These data confirm the old idea that cases which show the gametes for a long time during apyrexia are likely to relapse; but not because of any power inherent in the gametes themselves. The relapse is due, as I have stated, to a renewal of vitality on the part of the asexual generation that produces the crescents.

The hypothesis of Craig is open to many of the objections which have prevented that of Schaudinn from obtaining general acceptance. The actual conjugation of the young parasites has not been reported except by Mannaberg and Craig himself. Perhaps this is because to see the process requires more time and patience than many workers care to give. Henson<sup>1</sup> states: "I myself have observed the phenomena, if such it may be called, in both the tertian and estivo-autumnal infections," but does not state whether he observed it in fresh or stained blood. There is no doubt but that the forms described by Craig, in estivo-autumnal malaria, do occur. While I have never been able to witness actual conjugation in this infection, I have seen all the forms described by him as resulting from it.

Three years ago, while working with a very heavy estivo-autumnal infection, I noticed in the peripheral blood several parasites which did not correspond to the typical organisms of the sexual and asexual cycles. These parasites filled the erythrocytes containing them, stained somewhat like a crescent, but were round, not oval or semilunar in shape. They contained scattered pigment, and two to five well defined masses of chromatin. All stages of the asexual generation were present in considerable number in the serial slides taken over 48 hours, and it was very evident that the atypical parasites did not belong to any phase of the presegmenting *Plasmodia*. There were many well defined points of differentiation. The pigment was scattered and rod-shaped, not collected; the chromatin a much lighter red, and not so dense; and the cytoplasm

<sup>1</sup> *South. Med. Jour.*, 1911, 4, p. 130.

of a decidedly different tint. It was suggested that they might be quartans, but no other form of the quartan was found, and moreover, subsequent research showed that they were not of this species.

I did not see these parasites in the peripheral blood again for two years, when in one of the heaviest infections I have hitherto witnessed, I found them in great numbers. The patient was a little Negro baby, only three weeks old. No quinine had been given, and the baby died shortly after admission. In the peripheral blood were all ages of crescent formation and of the asexual cycle and many of the forms referred to, while the smears from the spleen at autopsy showed in very great number more different stages of the life-cycle than I had seen in any other case.

Slides were sent to Dr. Craig, who stated that the atypical parasites were identical with those described by him as resulting from conjugation, and he also very kindly gave me permission to quote him.

My own study of this case has convinced me that the forms mentioned were atypical developing crescents. Except for the distribution of the chromatin in many, and the shape, the other characteristics were the same. All stages of transition between these forms and crescents were observed. I have observed these same parasites at autopsy in several other cases, but always typical crescents were present also, and when both were sufficiently abundant, transitional stages were seen.

It is very evident from this that Dr. Craig has described correctly the forms which he interprets as resulting from conjugation in estivo-autumnal malaria, and others who have seen these slides agree that these forms were present in them. But neither in the spleen nor in the peripheral blood were many doubly infected erythrocytes, and I could not make out any difference between young gametes, except when these had the crescent shape, and young parasites of the "conjugation" type. Moreover, although all stages of sporulation of the asexual cycle were plainly visible, nothing resembling sporulation, except the scattered masses of chromatin, were seen in the "conjugation" forms.

If the form of parasite responsible for relapse is due to conjugation, then certainly fevers which relapse the more often should be those which have the greatest proportion of doubly infected erythrocytes. Now, it is well known that as a rule multiple infection of the erythrocytes is in direct proportion to the severity of the infection. But it is equally well known that the severity of the infection is no indication of relapse. The mildest quartan will

relapse time after time, and one may, as I have done, search in vain in such an infection for a doubly infected cell. As to this, it is Dr. Craig's opinion that it is possible to examine only an exceedingly small portion of the blood, and that doubly infected cells may be so scarce as to escape observation, although plentiful enough to furnish sufficient parasites to cause a relapse. This view has the merit of analogy, for one does not find parasites immediately after the bite of the infected mosquito, and those who believe that relapse is caused by a renewal of vitality in the asexual cycle admit that this cycle may at times be so few in number as to be unobserved in the peripheral blood.

If, however, relapse is due to any latent forms of parasite other than small numbers of the asexual cycle, such forms should be found sometimes in a careful search at autopsy in a country where latent malaria is known to exist in a considerable proportion of the population. Also, the peripheral blood should show these forms at times, when there are no clinical symptoms of malaria.

#### THE SECOND HYPOTHESIS.

Marchiafava and Bignami,<sup>1</sup> in their excellent treatise on malarial infection, called attention to the fact that relapses at short intervals could best be explained by assuming that a certain number of the parasites survived quinine treatment and the action of the protective forces of the body. This view, however, did not meet with general acceptance, because it was very generally thought that the asexual cycle, when its vitality was impaired, rapidly perished, for very often careful search in the peripheral blood between relapses failed to reveal parasites.

In 1910, Ross and Thomson<sup>2</sup> published the results of the first research wherein mathematical methods were used to count accurately the degree of infection. By use of the "thick film" method, they were able to show that although the usual method of blood examination might, and very frequently did fail to show parasites in the apyretic interval between relapses, in the thick films the organisms could be demonstrated in some cases during the entire interval. They also found that relapses so produced

<sup>1</sup> *Op. cit.*, p. 362.

<sup>2</sup> *Ibid.*

had no relation whatever to gametes or "resistant forms," but depended entirely on the degree to which the asexual parasites resisted quinine or the protective forces of the body. They<sup>1</sup> state:

"Moreover, the general trend of the curves suggests that they [parasites] were not found on these days [days in the middle of the apyretic period] only because their numbers were a little too few for detection. The parasite curve, at its height during a pyrexial period, generally falls very rapidly at first and more slowly later, and tends to reach its lowest about half-way between the two apyrexial periods. At this point it may or may not remain above the detectable limit [by thick film methods]. After this it was observed [by Thomson], especially in Cases 7, 17, 23, and 24, to begin mounting, slowly at first, until, when it reached the pyrogenic limit, another pyrexial period commenced. All this is scarcely compatible with the speculation that the apyrexial periods are due to the abrupt death of most of the asexual *Plasmodia*, or to their conversion into 'resting stages' etc. Nor do such speculations appear to be at all necessary. It is easy to see that the survival of comparatively small numbers of the asexual forms will suffice to keep the infection alive, not only for the short periods observed by us, but for 'relapses of long interval,' and for months and years."

These conclusions of Ross and Thomson, based as they are on accurate research of the highest degree, have been accepted, even by those who advocate some special form of the parasite as responsible for long interval relapses, as explaining relapses at short intervals. They are not accepted by Craig, Deaderick, and Henson as explaining long interval relapse.

In 1910, Bignami<sup>2</sup> published the result of his research for many years previously into the pathogenesis of relapse. He takes up in great detail the hypothesis of parthenogenesis, but unfortunately does not discuss that of intracorpuseular conjugation. In this paper, which ranks with those of Ross and Thomson in recent importance, after a careful examination of the claims made for a "resting stage" of the parasite, he concludes that there is no basis for such claims. He abandons, provisionally, his former hypothesis, by which he explained long interval relapses as due to merozoites which in some manner became invested with a protective coating, because after years of examination of autopsy specimens he could find no confirmation of his view. It is significant to note here that he, also, did not find any other form that could be interpreted as a "resting stage."

Bignami then explains relapse as due to the persistence of the asexual cycle, arguing from the viewpoint of analogy and biology.

<sup>1</sup> *Ibid.*

<sup>2</sup> *Op. cit.*

He believes that the asexual parasites become *immune* either to the effect of quinine, or to the protective forces of the body. This immunity may be complete or only partial. He cites as analogous to this view the recent experiments of Ehrlich<sup>1</sup> with trypanosomes. These showed that trypanosomes may be made immune against the protective forces of the body, against certain drugs, and against combinations of certain drugs, but that a strain immune against some is not immune against others. Experiments similar to these with other protozoa are also noted. He then cites the well-known immunity of malarial parasites in some instances to quinine. He concludes that this immunity, whether against quinine or against the protective forces of the body, will explain any case of relapse, whether at long or short intervals.

Three years ago, while working on the relation of the fever curve in malaria to the life-cycle of the parasites, I observed 50 cases in which quinine was not exhibited for some time. In a few of these only was it necessary to give the drug to control the fever, which disappeared spontaneously in most cases with rest in bed. It was noted then that many of the patients had parasites in the peripheral blood during apyrexia, and that an increase in the number of these always foretold a relapse. It was also observed that gametes were not always present, but when they were, there was no alteration in their morphology. The asexual parasites, however, underwent marked changes, and this without treatment. This fact led me to believe that the asexual cycle, having once lost its vitality, could not renew it, and I thought that a "resting stage" of some kind would be necessary before the infection could renew itself. Later, in studying quartan malaria, I thought that I had found such a stage.<sup>2</sup> Subsequent research, however, has convinced me that I was wrong, and that the forms which I took to be "latent parasites" were atypical stages of the life-cycle.

Not only did I observe ordinary relapse while studying the cases above mentioned, but I saw several cases of relapse with a species of parasite which replaced the one present when the patient was admitted. These cases were very carefully studied. The first infection, whether estivo-autumnal or tertian, was entirely replaced by the second infection. As one group increased in number, the other decreased. At this time Schaudinn's work on parthenogenesis, and Craig's conjugation hypothesis were known to me. I thought that since it was possible for me to observe relapse from the time when the parasites first appeared in the blood, and with a species different from the original infection, so that there could be no question of a renewal of vitality on the part of the latter, it would not be difficult to attribute the cause to one or the other hypothesis. However, I could find no verification of either, only asexual parasites were observed early in the secondary infections, and gametes of these did not appear until later. Neither did the original infections always disappear as gametes or "resistant forms," but often as disappearance of the asexual cycle. This fact was

<sup>1</sup> *Chemotherapeutische Trypanosomen-Studien*, Leipzig.

<sup>2</sup> *Proc. Canal Zone Med. Soc.*, 1910, 3.

particularly noted. Of course, some of the cases showed gametes after the disappearance of the asexual forms, but this was not a constant feature.

Having in mind Marchiafava and Bignami's description of double tertian and double and triple quartan cases that relapse in the original form, I sought for these also. In this I was successful, and observed from the time of admission, through an apyretic period in which the parasites disappeared (I was using the thin film method), relapse in two cases of triple quartan and one of double quartan, several of double tertian, and one of mixed estivo-autumnal and quartan. All of these relapsed in the form of the first observed infection; the triple quartan as triple quartan, the double quartan as double quartan, etc.

The mixed estivo-autumnal and quartan case is of particular interest. The patient was a little Columbian boy, about seven years old, who had come from Cartagena to Colon about a year before his admission to this hospital. He was admitted to the service of Dr. A. B. Herrick, chief of Surgical Clinic, for Hirschsprung's disease. Pending operation, he was placed in my ward. His blood examination on admission was negative for malarial parasites. About a week after admission, he had a chill, and manifested a rise in temperature to about 103. The blood examination showed a moderate single quartan infection. On the following day a heavy estivo-autumnal infection was also seen, and in proportion as this increased the quartan infection disappeared, so that two days later only a few quartan gametes were seen. Quinine was given, and the fever promptly stopped. Two weeks later the operation was successfully performed. About seven months later, the patient having in the meantime moved to a different locality, he returned for further observation. While in the ward he again developed fever, with quartan parasites, which were followed two days later by estivo-autumnal organisms.

The mother of this boy, a woman of exceptional intelligence for her class, is employed as a school mistress by the Panama government. She told me that before coming to Panama, her son had repeatedly suffered from quartan fever, and she described very accurately the quartan symptoms, and called them by name. It would appear, then, that the first quartan attack was a relapse. The estivo-autumnal attack may or may not have been primary. The second attacks of the two, following in the same sequence as at first, were very likely due to a relapse of each species, as quartan malaria is very rare indeed relatively in Panama, and the patient had not lived in the same locality as before the first attack.

I have also, for the past two years, sought especially for adult conjugation and parthenogenetic forms in the peripheral blood. These are very rarely encountered, and only as I have described above. They are almost always in relation to relapse or reinfection, as they are found, as I stated, only in anemic blood, and there was a history of previous malaria in all cases but one, that of the three-weeks-old baby.

Finally, in an effort to find the form of parasite latent in the internal circulation, I examined smears from over 200 autopsies, 150 of which were consecutive. Several times I found parasites when none had been encountered in the peripheral blood, and the cause of death was other than malaria. There were also cases of death from traumatism, in which smears from the spleen and rib marrow showed parasites. This work will be reported later in full, but I can state at present that at no time did I find any parasites other than forms of the sexual or asexual cycles, and these in about equal numbers. Nothing that I myself would call a "resting form" was seen, except the parasites that Dr. Craig interprets as conjugation forms, and that I think are atypical crescents.

## III.

In presenting the foregoing account of the various hypotheses as to the etiology of relapse, although I have stated my own opinions of their validity, I have tried to give impartially the findings of various workers. It is now my purpose to examine these hypotheses particularly with reference to their biological significance, in order to ascertain what relation they bear to observed facts in the life-history of the *Plasmodia*.

These facts I have stated previously. They are, briefly:

1. Relapse is a common feature of malarial infections.
2. Relapses follow most often, in the order named: untreated primary infections; insufficiently treated infections; and infections treated late in their course. Although rarely, they follow the most vigorously treated primary infections. And it is easier to effect a cure in uncomplicated than in complicated infections.

From these data it is evident that the cause of relapse is intimately connected with two factors in the life-cycle of the *Plasmodia*: (a) a stage when quinine as usually given does not eradicate the infection; and (b) this stage is in intimate relation to the time the infection has persisted. Any hypothesis that does not accord with these factors cannot be accepted unless it be admitted that the factors are false. Also, a hypothesis should agree with the clinical observations in relapse which so many careful workers have verified.

If it be admitted that parthenogenesis actually happens, it by no means follows that this is the cause of relapse. The evidence adduced shows that in all reported cases of the phenomenon except that of Schaudinn, and this is doubtful, an active asexual cycle was persisting at the same time, and further, that the parthenogenesis occurred during the progress of the relapse, and not at the beginning. It is necessary, then, to prove beyond question that there has been no confusion of parthenogenetic forms with atypical segmentation. The latter is known to occur, while the former, it may be said in all fairness, are somewhat arbitrarily classified, as Bignami<sup>1</sup> has pointed out. Ross<sup>2</sup> has very carefully reviewed the evidence brought forward by Schaudinn, and states:

"A patient who had long suffered from *P. vivax* (the tertian parasite) was attacked on the 29th of April and the 1st of May, and was found to contain both sporoids (asexual parasites) and gametids (gametes). A rally (period of intermission between relapses) now occurred, during which daily examination disclosed *varying* numbers of gametids only. On the 25th of May these parasites were *more plentiful*. Next day curious changes were noted in the female gametids, suggesting that they were producing

<sup>1</sup> *Op. cit.*

<sup>2</sup> *The Prevention of Malaria*, New York, 1910, p. 112.



spores similar to those ordinarily produced by the asexual sporoids. The author [Schaudinn] considered this to have occurred independently of fertilization by the male gametids, and to be due to parthenogenesis. The same day (26th of May) the patient had a slight rise of temperature to  $38.4^{\circ}\text{C}.$ , and in the evening ordinary young sporoids were found. Next day *only* these forms occurred. On the 28th of May there was a typical attack with temperature reaching  $40.75^{\circ}\text{C}.$ , and with the usual sporoids. The author carefully described the parthenogenetic forms, and traced the corresponding changes in the nucleus. . . . .

"I note especially that between the 1st and 25th of May the number of gametids *varied*, and was increased on the latter date. But this variation suggests that they were being produced all the time. The gametids are supposed to be produced from ordinary spores; so that we are forced to infer that a number of sporoids, some of them generating gametes, were present in the patient's body, although they were too few to be detected in the small quantities of blood examined by the author. On the 25th of May they probably increased in number sufficiently to produce a slight attack of fever, and then were mistaken for parthenogenetic gametes. On the 28th of May they produced a typical attack; and that is all. The supposed nuclear changes were reported on evidence of no great value. The cells were not actually observed undergoing the development which the author describes. He merely inferred the existence of the development from a study of different cells in what he thought were different stages of that development."

Now, in tertian malaria, very often the entire cycle of development of the gametes can be followed in the peripheral blood, and is completed in from four to five days. Continued persistence of the gametes for longer than this period, as Ross notes, is a sure indication of the persistence also of an asexual cycle. Adult tertian gametes do not persist as individuals for 26 days. For if quinine be exhibited in full doses, the gametes, no matter how plentiful at first, will disappear in four or five days. I have demonstrated this by many experiments, working both with thin and thick films. If the gametes persist longer than this period, it means that the quinine is not being properly absorbed, and it should be given hypodermically or intravenously; either method, when properly administered (of which more later) will cause prompt disappearance. Nor does the fact that in Schaudinn's case the gametes disappeared after the sporulation of May 28th prove that such disappearance was due to their sporulation. In untreated tertian malaria, after the asexual parasites have disappeared for a while from the peripheral blood through the action of the agencies which bring about the so-called spontaneous cure, it is not at all unusual to observe a similar disappearance of the gametes a short time later.

In view of these facts, and our recent knowledge that when the thick film method is used the asexual parasites do not disappear all at once in untreated malaria as in the case reported by Schaudinn, not only is it impossible to exclude the presence of an asexual cycle at the time when the clinical symptoms of relapse were manifested, but the probability is that such a cycle was present, as Ross notes.

Relapse is a common feature of malarial infections. Mannaberg<sup>1</sup> notes that "malaria is one of those infectious diseases in which a relapse may be considered an essential feature," and goes on to state that "it makes little difference whether the first disease (attack) recovered spontaneously or was cured by the action of quinine." In passing, it may be observed that Mannaberg's opinion does not speak well for the methods of treatment used against malaria at the time when he wrote. Now, to explain the rarity with which parthenogenetic and "latent" types are observed, when the result of their further development is so common, it is assumed by those who postulate the necessity for such types of the malarial parasites that in the resting stage these types remain in the internal circulation, and further, that in most instances their sporulation takes place in the internal circulation, so that neither the types nor their further development are often seen in the peripheral blood.

But there are several other types of the *Plasmodia* that are rarely found in the peripheral blood; particularly certain phases of the estivo-autumnal parasites—the young gametes, and segmenting and presegmenting forms. Yet these are often to be found in abundance in the spleen and bone marrow at autopsy, after death from pernicious malaria, and in malarious countries are not infrequent at autopsy when death has occurred from a cause not at all connected with malarial infection. Craig reports seven cases of the latter—three of tertian, and four of estivo-autumnal infection—and notes that he did not find gametes. Bignami<sup>2</sup> also reports such cases, and I have observed quite a number myself. Dr. S. T. Darling and Dr. H. C. Clark, in their extensive autopsy experience here, inform me that this occurrence is

<sup>1</sup> *Op. cit.*, p. 340.

<sup>2</sup> *Op. cit.*

not at all infrequent. However, none of the authorities mentioned, nor have I myself, found at autopsy in acute or latent malaria any type of parasite other than the normal forms of the life-cycle according to our interpretation, except the forms previously referred to, that Craig holds to be the result of conjugation, and I believe to be developing crescents. It may be argued as has been noted, that parthenogenetic and "latent" forms are too few to be observed when the infection is latent, or that they had all sporulated, so that only the normal forms of the life-cycle were observed in the instances referred to above. But such a supposition is based upon speculation, and not upon actual observation, and, until supported by positive data, may not be accepted.

Parthenogenesis will not explain why triple quartan and double tertian infections relapse as such. Although many cases of malaria relapse at definite intervals, such as 7, 14, or 21 days, many more relapse at irregular intervals, as is very well shown by Craig<sup>1</sup> in his careful study, and no definite period of latency can be predicated. If it is difficult to explain the mechanism by which any latent infection is awakened, it is much more so to infer that gametes and "latent" forms renew an infection by sporulation at intervals of 24 hours, and so start again the cycles of triple and double infections.

It is admitted that if the continued persistence of gametes were due to an inherent longevity of these as individuals, parthenogenesis as a factor in the etiology of relapse could be accepted. But a definite life-cycle for gametes has been demonstrated. They originate in the asexual cycle, and their continued persistence over a period longer than 10 days or two weeks in estivo-autumnal malaria means a continued persistence of the asexual cycle that produces them. The cycle of the tertian gametes is, as I have noted, from four to five days. I have also worked out the cycle of the gametes in quartan malaria.<sup>2</sup> It takes place in six days, after which, in properly treated cases, or in cases where there is a spontaneous disappearance of the asexual forms, the gametes rapidly disappear.

<sup>1</sup> *The Malarial Fevers*, New York, 1909, p. 161.

<sup>2</sup> *Op. cit.*

As has been stated, the gametes are not definite factors in relapse. This, as is well known, occurs quite independently of the production of sexual parasites, whether in the peripheral blood or in the internal circulation, as far as the observation of many authorities and my own work is a criterion. If the gametes did not have a definite life-cycle, but persisted indefinitely, independently of the fate of the asexual cycle, if parthenogenesis were the etiological cause, since it is well known that adult gametes apparently are not affected by quinine, then treated cases in which the gametes are the latest forms of the parasites observed should always relapse. But many cases of primary and relapsing infections, in which the gametes were the last forms seen, have been permanently cured by prolonged and vigorous treatment with quinine, and in a time well within the limit of observed periods of relapse. The observations of Ross and Thomson<sup>1</sup> on relapsing cases in Liverpool, and many on primary and relapsing cases in this hospital, have shown that under proper treatment the crescents and other gametes will disappear within the limits of the life-cycles assigned to them.

If these facts are true, there can be no doubt but that the hypothesis of parthenogenesis does not accord with the factors common to relapse, which have been noted previously. While the older gametes appear to be immune against quinine, there is no proof that they persist as individuals for the long periods of latency that are recorded, some of them for over two years, nor, as has been noted, is the formation of gametes in relation to the observed factors in the etiology of relapse.

Many of those who uphold the single observation of Schaudinn, and who regard it, as Bignami states, "as though it were a datum of fact at this time unassailable" would do well to consider the criticism of Ross and of Bignami on this point. They would do well, also, to remember that the supposed confirmations of Schaudinn's work, when carefully analyzed, refer much more to a process that took place during the course of a relapse, rather than to a process that occurred at the beginning of one.

<sup>1</sup> *Ann. Trop. Med. and Parasitol.*, 1910, 4, p. 137.

The conjugation hypothesis of Craig<sup>1</sup> is founded on far more sufficient data and observation than that of Schaudinn. Here is a problem of interpretation of process and resultant forms, and a concordance of these with known clinical and biological factors in relapse, rather than morphological description of a doubtful sporulation of gametes at a time when a normal asexual cycle must have been in progress. Craig's study covered a period of nearly seven years, and he states that he observed conjugation in about 600 cases. He claims for the process that it results in the union of the nuclei of two young, unpigmented parasites, with subsequent reduction of chromatin, and a union of cytoplasm as well. He states that conjugation "occurs only after the plasmodia have multiplied in the usual manner for several generations," and that it is observed only in those malarial infections which have persisted for some time, adding that it never "occurs during the first few days of a malarial infection," and further, "I have never observed the process in cases in which but two or three paroxysms had occurred prior to treatment, but after the infection has persisted for a week or more, intracorpuseular conjugation is invariably observed." Neither does the phenomenon exhibit itself when quinine has been administered in sufficient doses promptly on the appearance of the symptoms of the primary or of the recurrent attacks. It is especially frequent when parasites are abundant, and there are many doubly infected cells, but may be found in any infection that has persisted for some time, if the parasites are abundant enough to allow of extended observations.

It has been argued against Craig's hypothesis that he has mistaken double infection of the erythrocyte in the early period of the asexual cycle for conjugation. In my experience, this criticism is not altogether well founded. The larger forms, described by Craig as the result of conjugation, certainly bear no resemblance, in my opinion, to doubly infected cells. His interpretation of the appearances in stained specimens, described by him as the beginning of conjugation, is, however, a matter for argument. And in differing with him in some respects as to this interpretation, as I shall do, it is well to remember that his experi-

<sup>1</sup> *Jour. Infect. Dis.*, 1910, 7, p. 285.

ence with all aspects of malaria and the *Plasmodia* is that of one who has made a careful study for many years.

As far as I can ascertain, no one has hitherto attempted to confirm or to disprove Craig's hypothesis by repeating his observations to any extent and for a sufficient time. Such confirmation or denial as has come to my notice has been based rather on a priori reasoning than on careful study. On this account I shall state only the conclusions which I have reached as the result of three years' study on this hypothesis. It is proper, I believe, to mention here that Dr. Craig, in a recent personal communication, states that he is of the opinion that more than one factor in the cycle of the parasites enters into the etiology of relapse, and while he believes that short term relapses are explained by assuming a continuation of the asexual cycle, he does not agree that such an assumption will explain relapses at long intervals.

In my study, I have never seen actual conjugation of living parasites. This does not, however, in my opinion preclude the possibility of such a process. Conjugation, according to Craig, by no means follows every double infection of an erythrocyte, and is a phenomenon that requires several hours for its completion; so that the difficulty of observing the entire occurrence is obvious, when it is remembered that two parasites may lie in apposition for an hour or more, and then separate. By the time one has made several such observations, the cycle has passed the stage when conjugation takes place. Craig<sup>1</sup> and Mannaberg,<sup>2</sup> however, state positively that they have witnessed the entire phenomenon, and their descriptions are very convincing.

In stained specimens I have repeatedly seen many of the forms described by Craig as various stages during conjugation. But when I attempted to find these in the fresh specimen immediately afterward, I was not successful, as I observed only different aspects of multiple infection of the erythrocytes. It is a matter of common occurrence to see in stained specimens of any heavy estivo-autumnal infection the forms described as the immediate result of conjugation, and similar forms can be seen in a control fresh specimen, as far as

<sup>1</sup> *Op. cit.*, p. 301.

<sup>2</sup> *Ibid.*, p. 300. See also "Malarial Diseases," Nothnagel's *Encyclopedia of Practical Medicine*, Philadelphia, 1905, p. 52.

the appearance of the parasite is a criterion. Forms with two "vacuoles" separated by a band of cytoplasm, and with a common periphery, I have repeatedly observed under the conditions when conjugation should take place, but after being watched for a while, such forms always returned to the original ring shape, and, indeed, not infrequently reverted to the first-described condition.

In untreated estivo-autumnal infections I have never observed any but normal forms of the asexual cycle. Only in tertian and quartan parasites have I seen the atypical segmentation mentioned. I have followed in the peripheral blood more than once a complete cycle of the estivo-autumnal parasites, in the beginning of which there was, in stained specimens, every appearance, as far as I could judge, of conjugation; but the later phases of the cycle showed only forms which I interpreted as normal, nor were there atypical delayed parasites, such as one would have expected had conjugation occurred. As to the adult forms mentioned previously, I interpret them as rather atypical developing crescents, and these Dr. Craig interprets as the result of conjugation.

Further, at no time in my study of tertian and quartan infections could I demonstrate any similitude of conjugation. This was seen by me only in estivo-autumnal infections. From the account given by Craig of the large tertian parasites resulting from conjugation, and from the appearance of these in the photographs he presents, I feel sure that such parasites are the same as those I have described as resulting from atypical segmentation, and similar to the parthenogenetic forms of Schaudinn, especially Craig's Fig. 9, which he himself thinks is similar to a so-called parthenogenetic form, as he<sup>1</sup> states: "This is the form which may have been considered by Schaudinn as a macrogamete undergoing parthenogenesis." It is very true that Craig's pictures are, as he claims, easily differentiated from normal segmenting and presegmenting parasites, but whether they are different from the ones which I hold to be the result of atypical development is something that can be determined only by other workers.

I have seen very many such forms toward the close of tertian and quartan cycles, and particularly in the latter instance, when a

<sup>1</sup> *Jour. Infect. Dis.*, 1910, 7, p. 311.

very careful study of the earlier stages had shown but few, if any, doubly infected cells, or forms resembling the phases of conjugation. For this reason especially I believe that the forms in question are atypical, and not the result of conjugation. In tertian and quartan infections such parasites are also seen at times when the rest of the cycle is advanced or delayed, thus lending color to Craig's belief that conjugating forms do not accompany the rest of the cycle in development; but since double infection is so common in these infections in the Canal Zone, it would not be possible to say that these parasites did not belong to a second cycle.

From the viewpoint of morphology, I am convinced that the forms as described by Craig in stained specimens for the actual conjugation and further development of malarial parasites do exist. The difference of opinion as to the precise classification of these forms is, however, a matter of interpretation, and as such I leave it.

The relation of these forms to the known factors in relapse should be carefully investigated. If they occur, as Craig states (and in this respect my findings corroborate his), only after the infection has continued for some time, then the hypothesis offers an explanation of the relapses that so often follow infections not treated until the clinical symptoms have been well established. Unfortunately, Craig could not follow the complete cycle of his forms, and those that he describes as resembling segmentation were not found at the beginning of relapse, but later in the infection, after conjugation had been established.

And here I would again take exception to the acceptance of sporulation which is claimed to result from parasites other than those of the asexual cycle, when such sporulation is found during the febrile stage, or at the close of it. If any latent form of the parasite, whether a gamete or the result of conjugation, which does not pertain to the asexual cycle, by its sporulation brings about a relapse, such sporulation should be observed at the beginning of the renewed cycle of the brood of parasites that is responsible for relapse, and not at the height of activity or at the close of such a cycle. Any sporulation observed under the latter conditions is a phenomenon that can be interpreted only as happening during



ordinary schizogony, and bears no relation to the etiology of relapse, as Bignami well observes, no matter how rare or peculiar the form in question. Unless such "latent" forms are so plentiful that one period of sporulation would determine the symptoms of relapse, in which case certainly the process should be observed in the peripheral blood, the parasites must increase through several generations before symptoms are evident, and such parasites as are seen during the pyrexia, or at the close of it, are of necessity products of those whose awakening from latency caused the relapse. Yet various authors who describe the sporulation of "latent" forms find these, not at the beginning of the schizogony that ultimately results in the clinical manifestations of relapse, but during the period of such schizogony, or at its close, and claim that such parasites are those which initiate the relapse. Such reasoning describes effect for cause. Nor does it aid the argument to assume that when the forms are found at the end of the relapse they are those which will continue the infection, for there are no facts to prove this assumption but only conjectures.

The increase of parasites resulting ultimately in the clinical manifestations of relapse, from the time these appear in the peripheral blood to the period of greatest number and through decline, has been carefully studied by Ross and Thomson<sup>1</sup> in a number of cases, and I have done the same. We have found nothing other than the normal sporogony and schizogony during this increase and decline (except the atypical forms as noted), nor have we found any sporulation of "latent" forms at the beginning, or development of these at the end. If it be argued that the "latent" forms, at the beginning of renewed vitality, are in the internal circulation, or are so few in number as to escape notice, then again the hypothesis rests upon conjecture, and not upon established fact. For careful examination of autopsy preparations when latent malaria has been concurrent with the cause of death, and when parasites were not found in the peripheral blood, even after very careful examinations, has failed to demonstrate any form of *Plasmodia* other than those I hold to pertain to the sexual and asexual cycles.

<sup>1</sup> *Op. cit.*

Conjugation, which is admitted to occur only late in the infection, will not explain many cases of relapse following only one paroxysm, nor relapses following infections in which the process was not observed. While some authors hold that the more severe the infection, and the longer it persists without treatment, the more likely is relapse to follow, I do not agree altogether with this belief. Certainly, clinical observations agree that untreated infections which persist over a long time are very prone to relapse, but very slight tertian infections often relapse after only one or two mild paroxysms, when the parasites have been very scanty indeed, and the quartan infection is notorious for its relapses, without regard to the severity of the infection and the number of parasites, or the duration of the disease.

However, it must be admitted by any impartial student of malaria that the hypothesis of conjugation itself, whatever may be the interpretation of the process, rests on the careful and extended observations of an experienced authority, and can be confirmed or denied only by a study as careful as his.

#### IV.

In the introduction to this paper I gave as my definition of relapse:

"A return of the fever and parasites after the former has ceased and the latter have disappeared from the peripheral blood, reinfection being excluded. I do not include those cases in which quinine has not been administered in doses sufficient to clear the peripheral blood of parasites. Such cases are not true relapses, for the original infections are only diminished."

So widespread is the belief that there are two kinds of relapses—one due to a simple renewal of vitality at short intervals, without further change of the asexual generation; and the other, to a renewal of vitality at long intervals of some "resistant" or "latent" form, derived from the asexual cycle—that in order to keep clearly in mind the various hypotheses as to the etiology of relapse I have not as yet modified my original definition. The result of my studies for the past three years, however, has convinced me that there is but one primary cause of relapse, a renewal of vitality on the part of the asexual cycle, and that this is true, whether relapse

takes place a week or a year after the original infection has subsided. From this viewpoint a relapse is not an occurrence apart from the original infection, but merely a phenomenon manifested at different times by an infection that has not been eradicated, in so far as the symptoms of chill and fever are concerned, and as such I wish to consider it in the remainder of this paper.

The relation between the numerical quantity of the infection with its toxic products, and the reaction of the human organism as manifested by pyrexial symptoms, varies so greatly that the amount of infection which produces definite febrile attacks in one individual may show itself in another as a non-febrile cachexia, and so be overlooked. Clinically, then, while a relapse may be defined as a return of the febrile symptoms due to an increase in the number of parasites, biologically it is the response of the asexual cycle to a renewal of vitality by which the number of organisms again increases, and as such may appear as a return of fever, as cachexia, as neuritis, or as one or more of the many diverse symptoms associated with what is called "chronic malaria." This latter term, it may be noted, means no more than that the primary infection has not been eradicated. The wave-like rise and fall in number and activity, with lower and higher levels that remain stationary over varying periods, is a phenomenon common to the cycles of many protozoa, free living as well as pathogenic, and the latter has been particularly well shown by Ross and Thomson<sup>1</sup> in their study of a case of human trypanosomiasis.

As long as the malarial infection persists, one may expect at times some clinical manifestation of it, and what is commonly termed a relapse—recurrent pyrexial symptoms at varying intervals after the primary manifestations have subsided—is, as noted, no more than one of the many responses of the human organism to the products of an increased number of parasites following the renewal of vitality in the asexual forms residual after the primary attack, or after each succeeding pyrexia.

The hypothesis that the asexual generation alone, and without alteration other than increase and decrease in its reproductive power, is responsible for relapse as I have defined it above, is upheld

<sup>1</sup> *Ann. Trop. Med. and Parasitol.*, 1911, 4, p. 261.

recently by Bignami and by Ross and Thomson, and the reasons for their belief have been given elsewhere in this paper. To these I shall now add the conclusions to which I have come as the result of my own study.

To substantiate the hypothesis that the asexual generation is the only phase in the life-cycle of the *Plasmodia* which is the etiological factor in relapse, it is necessary to assume, as far as can be done by the proof at hand, two propositions:

1. That the asexual cycle is endowed with a potential of vitality which enables it to persist, at times in numbers too small to be detected in the peripheral blood, over a period of at least two years, corresponding to the longest known interval between a primary infection and a relapse, or between one relapse and another.

It may be noted here that much of the data as to the exact length of time between these phases of the malarial infection is very inaccurate, and extremely unsatisfactory. Anyone who has worked to any extent on the diagnosis of malaria will refuse to accept as positive an account of a malarial infection which is not satisfactorily verified by accurate blood findings, no matter how definite the clinical symptoms. Nor will an account of a relapse<sup>1</sup> after such an infection be accepted, unless it is shown beyond doubt that there was no chance of reinfection in the meantime; that parasites similar to those of the primary infection were found during the relapse; and that the individual who reports the relapse knows exactly what a malarial parasite looks like, whether in fresh blood or stained. These conditions are made necessary by the reports in recent literature which are not verified by sufficient data, and I am sure that no one who has worked for several years with fresh blood and stained, and with good stains and bad, will object to these restrictions. Under them, the longest period of relapse that I have been able to ascertain—two years—was reported some years ago by Marchiafava and Bignami.

2. That the asexual cycle can become relatively immune to the protective forces of the body, and also to the action of quinine.

1. The assumption that the asexual cycle can persist over long periods, although in very small numbers, is one that many authorities on malaria refuse to accept; and, indeed, it is not altogether susceptible of proof by direct methods. However, very many facts in support of this assumption can be legitimately adduced. It may be admitted, at the outset, that the same arguments which militate against an acceptance of the view that the asexual cycle renews itself by parthenogenesis, or by sporulation of "latent" forms when these are too few to be detected in the peripheral blood

<sup>1</sup> In this note I use the term "relapse" in the sense of the original definition.

or elsewhere, would also hold against the belief that this cycle renews itself from asexual forms which are also too few to be detected, were it not for the additional proof that can be brought forward, by analogy as well as by fact.

Analogy, as Bignami observes, while not of value unless supported by correlative data, takes on importance when such data are available.

The life-cycle of the *Plasmodia* in man may be compared to those of other pathogenic protozoa which also pass in man only one part of their existence. The best known of these are the *Leishmaniae*, the *Trypanosomata*, and the *Entamoebae*. *T. pallidum* as far as I know, goes through its entire life-cycle in man; so that the only pathogenic protozoa (if indeed they are protozoa), in which there is an extra-corporeal cycle, that show in the human organism "latent" or "resting" phases, are the spirilla forms responsible for relapsing fever, and in these there is no certainty as to such phases.

Now Ross and Thomson have shown by their precise methods that the continuance of trypanosome infection in the human body is due only to a persistence of the asexual phase. I am also informed by Dr. S. T. Darling,<sup>1</sup> who has made a careful study of the animal trypanosomiasis which he has found in the Canal Zone, that he has observed in infected animals none of the so-called "latent" bodies or "resistant" forms of the parasites. Similarly, I have failed to find any positive account of a "resting" stage, or a "latent" form of either *L. tropica* or *L. donovani* in the human body. In fact, one of the highest authorities on these species, Leonard Rogers,<sup>2</sup> states that only one phase is so found, that of the cycle of the non-flagellate bodies. Yet sleeping sickness, oriental sore, and kala-azar persist over long periods, and the infections are maintained, as far as I know, solely by the persistence of organisms that correspond to the asexual cycle of *Plasmodia*.

Amoebic dysentery is a protozoon infection well known for its tendency to "relapse" over many years. Like *Plasmodia*, *Entamoebae* produce forms, the cysts, apart from the asexual cycle,

<sup>1</sup>*Jour. Infect. Dis.*, 1911, 8, p. 467.

<sup>2</sup>*Fevers in the Tropics*, London, 1908, p. 85.

and these have an extra-corporeal existence, which is intended for the transfer of the infection to another host, although unlike in *Plasmodia*, these forms do not have to pass through another organism. Whether autogamy takes place in these cysts, so that they are self-fertilized before renewing the infection elsewhere, or whether there is in the intestinal canal conjugation of the young *Entamoebae* that Darling<sup>1</sup> has shown to be derived from these cysts, is disputed; but it is a fact that as far as is known at present, infection with *Entamoebae* can and does persist in man for many years, without any known cause for continuation other than the life of the asexual cycle of these parasites. There are, I am aware, observations on a supposed conjugation of the vegetative forms, but these have not been confirmed, nor do those who made them insist on their validity.

Free living protozoa, as shown by the experiments of Calkins<sup>2</sup> and of Woodruff,<sup>3</sup> when the environment is favorable, can reproduce themselves for many generations without necessity for parthenogenesis or for conjugation. Woodruff, repeating Calkins's original experiments, has carried *Paramecium* through an asexual cycle of over 3,000 generations.

These facts will serve to show that, in so far as analogy has value, there is no reason why the asexual cycle of the *Plasmodia* should not persist for a time equal to the longest recorded interval between a primary infection and a "relapse," or between one "relapse" and the succeeding one. I might add here again, for emphasis, that in the other pathogenic protozoa, as in *Plasmodia*, what has not infrequently been taken for a "latent" form has later been recognized as merely a product of degeneration.

To come now to more direct proof of the vitality of the asexual cycle of the malarial parasites, it can be shown that these do persist at times in sufficient quantity to be demonstrated in the peripheral blood, for apyretic periods that are equal to those which many observers claim to be too long for the persistence of this cycle. I myself have seen a quartan infection, that apparently neither

<sup>1</sup> *Arch. Int. Med.*, 1913, 11, p. 1.

<sup>2</sup> *Op. cit.*

<sup>3</sup> *Jour Am. Med. Assn.*, 1912, 59, p. 1724.

increased nor decreased in quantity, show itself daily in the peripheral blood for a period of six weeks, during which there was a considerable period of apyrexia. It might have run on in this way much longer had it not been killed by large doses of quinine. Others, particularly Marchiafava and Bignami, have reported similar cases, which lasted in a like manner even longer. As for persistent negative findings in the peripheral blood, in the interval between febrile relapses, such as Thayer<sup>1</sup> in his excellent monograph puts such stress upon, it should be remembered that the "thin film" method, or else the examination of fresh blood, was used, and neither method can compare in such instances with the accuracy of the "thick film" method originated by Sir Ronald Ross.<sup>2</sup>

Nor do I think it is logical to assert, in view of the data which I shall now present, that the asexual cycle perishes in the intervals when even by the "thick film" method no parasites can be found in the peripheral blood. The finding of parasites in the peripheral blood means no more than that infection has attained to a certain numerical quantity, as Ross has pointed out.<sup>3</sup> If the infection maintains itself in this quantity for six or eight weeks, what reason is there to suppose that it cannot maintain itself in smaller numbers for the same, or even a longer period? The finding of the parasites in the peripheral blood is an indication of a high potential of vitality; there are no grounds for supposing that a lower potential may not be in force without such manifestation.

That the infection may persist in small numbers is, I believe, sufficiently clear, when one considers the course of what is called "chronic malaria." Marchiafava and Bignami make a distinction of value with reference to that term.

On the one hand, are the cases in which the fever is mild, and the pyrexial attacks are infrequent. The only symptoms of such cases are those referable to the acute attacks. On the other, are cases in which the febrile relapses may be mild or severe, and occurring with more or less frequency, but in which appear the well-

<sup>1</sup> *Lectures on the Malarial Fevers*, New York, 1901, p. 184.

<sup>2</sup> *Op. cit.*, p. 91; also *South. Med. Jour.*, 1911, 4, p. 688 and p. 725.

<sup>3</sup> *Op. cit.*, p. 115.

known symptoms of chronic malaria—anemia, enlargement of the spleen and liver, edema, palpitation, and dropsy.

Now, the symptoms of this second group are not brought about solely by the effects of four or five febrile attacks, unless these are prolonged and of unusual severity. For many persons who, by reason of reinfection or relapse have had a similar number of febrile attacks, when these have been treated promptly and energetically, show no further disturbance than that associated with the attacks themselves. The other symptoms often develop in the apyretic intervals between the febrile manifestations, even when the latter are mild, and are due to an additional factor. This factor, even before the hypothesis that the asexual cycle persists between febrile relapses was advocated, Mannaberg looked on as "an obstinate persistence of the virus"; and Marchiafava and Bignami defined chronic malaria: "When the infection of the organism continues for months and even years." It is clear from a study of their work that these authorities recognized a persistence of the infection, but at that time they did not correlate this persistence with that which occasions relapses after long apyretic intervals.

But the variation in intensity of malarial infection is one of degree only, and such infection continuing over a long period of time does not of necessity always produce pyrexia, or even other symptoms.

In the medical service of Ancon Hospital, Dr. W. E. Deeks years ago recognized post-malarial symptoms such as anemia, neuritis, cephalgia, and minor complaints occurring at more or less frequent intervals after the primary infection, or between relapses, when pyrexia was absent. And he found that such cases often do not improve on quinine by mouth, but clear up rapidly after two or three hypodermic injections of quinine.

There is also a group of cases similar to these, but further advanced, in which the anemia is more profound, and gastrointestinal disturbances and other signs of toxemia are more apparent. These cases also may resist quinine by the mouth, during apyretic intervals, yet the condition yields rapidly to hypodermic or intravenous administration. Similar cases are not uncommon,



with and without the finding of parasites in the peripheral blood. Through the courtesy of Dr. D. M. Molloy, of the Philippine General Hospital, I can present the details of a very instructive case:†

The case was that of an American civil engineer, aged 23, who contracted an estivo-autumnal infection while in the Philippines, in July, 1911. He was treated in the hospital with quinine by mouth until the infection was controlled. He left the hospital in good condition, *but still had a few crescents in his peripheral circulation.*

In September he returned to the hospital, "all run down," as he expressed it, but had had no paroxysms during the interval, and had no fever at the time of this admission. The patient was almost cachectic, there was marked anemia, and again the blood examination showed *a few crescents.* This time he was given daily doses of hydrochlorid of quinine hypodermically, 1 gram, later supplemented by 1.6 grams by the mouth, and also iron and arsenic tonic. Improvement was slow, and the patient remained in hospital for two weeks. No parasites were found on discharge.

In December he returned to hospital in a worse condition than before, although in the meantime he had had no recurrence of the febrile attacks, and had consistently taken the anti-malarial tonic prescribed. Blood examination *still showed crescents.* The patient was so weak that he could not perform his duties, and had lost much flesh. Some edema of the extremities had developed, and there was present a marked secondary anemia.

He was now given 1.3 grams of the bihydrochlorid of quinine and urea intravenously each day for three days; then 2 grams every other day for a week; and then the latter dose every third day for another week. The drug was given in 200 c.c. of normal salt solution.

The improvement in his condition, Dr. Molloy notes, was so rapid as to be almost marvelous. No crescents were found after the third day, and the patient gained 16 pounds during the three weeks that he was in hospital. Subsequent blood examinations were made monthly until July, 1912, and parasites were not found again, while the patient has remained in good health.

Only to the persistence of an asexual cycle from July to December could the symptoms be attributed, because no one claims that the gametes themselves have any ill effect on the human body.

Between the mildest cases of chronic malaria and those I have just cited, and between the latter and the severest cases with enlargement of the spleen and liver, profound anemia, edema, and other indications of great organic disturbance, are all grades of transition, so that no sharp distinction can be made. And such cases, as noted, occur with and without the finding of parasites in the peripheral blood, and with and without subjective pyrexia

† The following case is cited almost verbatim from the notes sent by Dr. Molloy, and I wish to take this opportunity to thank him for the report.

at intervals. Probably a carefully kept temperature record would show varying degrees of pyrexia at some time in all such cases, as Mannaberg, and Marchiafava and Bignami believe. Yet any of these symptoms, except those due to lesions beyond repair, will yield to proper and continued quinine treatment. And since the clinical manifestations of "the obstinate persistence of the virus" are so varied in degree, it is a logical inference that the "virus" itself also varies in quantity and virulence. For by no hypothesis of "resting stages" or of "latent" parasites can be explained the continuation of the symptoms during the apyrexial periods, with prompt disappearance after a suitable administration of quinine. Only the persistence of an asexual cycle of the *Plasmodia*, with the formation of toxin and direct and indirect destruction of the erythrocytes, will explain such a clinical picture. I believe that there is abundant evidence in the above data to demonstrate that it is possible for the asexual cycle to persist over several months without giving rise to subjective febrile symptoms, even when the parasites are not found in the peripheral blood.

Further, it by no means follows that the asexual cycle of the *Plasmodia* is absent from the internal circulation because it cannot be found in the peripheral blood after repeated and careful examination. More than once, in diseases such as abscess of the liver, or septicemia, at the onset or on admission to hospital it has seemed as though malaria were a factor, although careful examinations of the blood have been made here repeatedly with negative findings. Yet at autopsy forms of the asexual cycle were found with more or less frequency in smears from the spleen and marrow. Also, in cancer, chronic nephritis, and similar diseases of long duration, when the patients were in hospital several months under observation without any symptoms of malaria and without any findings of parasites in the peripheral blood, we have found these at autopsy in the spleen and marrow, in forms of the asexual cycle.

As I have previously noted, I do not believe that these findings are the result of sporulation of gametes or of "resting" stages. In cases of latent malaria I have never found at autopsy either gametes or what might be called "resting" stages of tertian or quartan infections, and if such forms were present, it is very

difficult to understand why they should have been repeatedly overlooked.

2. The demonstration that the malarial parasites of the asexual cycle may become immune to the protective forces of the body is self-evident from a consideration of any untreated case of malaria in which they persist for a week or more, or indeed from the fact that they persist at all, and need not be discussed further.

The effect of quinine on the *Plasmodia* is, however, so intimately related to complex factors in absorption, as to make difficult or impossible at this time a definite statement as to the precise degree of immunity that the parasites may acquire. Direct observation shows there must be some immunity so acquired. Ross and Thomson<sup>1</sup> describe a remarkable case of double infection with tertian and estivo-autumnal parasites which plainly shows such immunity.

The patient was admitted on the 23d of October, after having been ill at intervals for 159 days, during which time he had taken quinine thrice daily for a month, and at other intervals as well. On admission he had fever, and a double infection with gametes and asexual forms of *P. falciparum*, and with asexual forms of *P. vivax*, was found. Quinine hydrobromid in liquid form, in doses of 10 grains three times a day, was given, and continued for 17 days, during which period the temperature remained normal. No asexual parasites were found after five days.

From the 3d until the 9th of November, the blood was not examined. On the 10th there was a rise in temperature, the blood was examined, and no parasites were found. The quinine was discontinued. The temperature of a true malarial type, persisted, however, and on the 14th another blood examination was made, this time showing parasites of both species, and gametes of *P. falciparum* also began to appear. From the 14th to the 18th of November quinine was given in doses of 20 grains per day. However, the fever continued, and the parasites increased in number from a few on the 14th to 18,000 per cubic millimeter on the 21st. On the 22d after the dosage had been increased to 30 grains per day, an extra dose of 30 grains was given intramuscularly, and 12 grains of methylene blue in pill form was given also daily. In three days no asexual parasites could be found, but the crescents persisted for 14 days longer or about three days after their normal life-cycle, which would have been expected.

It was supposed that perhaps the quinine might not have been properly absorbed, but a urinalysis made by Dr. G. C. Simpson showed that such was not the case, for the patient was excreting 13 grains a day out of the 30 administered, which, in the author's opinion, was the usual amount.

Bignami<sup>2</sup> reports a similar case of Torti's and Angelini's, when after a gram of quinine had been given by hypodermic

<sup>1</sup> *Ann. Trop. Med and Parasitol.*, 1912, 5, p. 539.

<sup>2</sup> *Op. cit.*

daily for a month, a relapse, with the finding of parasites in the blood, followed almost immediately. Dr. Molloy's case also shows an acquired immunity against quinine on the part of some of the asexual parasites.

Cases very similar to these have been observed here not infrequently. Dr. Molloy states that he has seen relapse even after intravenous injections. I have observed one such case in which it was possible to say that the attack was a relapse. As far as I can determine, cases that relapse after intravenous doses of quinine, are those in which small doses have been given, or that have been treated with quinine by mouth previously. Mannaberg<sup>1</sup> is of the opinion that it is difficult to attack organisms in the parenchyma of the spleen and marrow with the drug, and I think that there are very good reasons for this belief.

For, in cases dying from malaria after three or four days of vigorous treatment, very often it is only in the spleen and marrow that parasites can be found. Several times at autopsy I have seen a large number of parasites in the placenta, after three or four days' treatment, when the peripheral blood was negative after prolonged search, and when but few parasites were found in the spleen and marrow. Smears of the spleen and marrow contain relatively a much smaller number of erythrocytes (in which the asexual cycle lives) than do smears of the peripheral blood, so that when parasites are found without trouble in the former when the latter is negative, it is obvious that in the former, for some reason, the parasites have not been affected as in the latter. In fact, in the spleen and marrow not a few of the parasites appear normal, although most of them show the effects of the quinine.

I have also noted, in following at autopsy cases that have died of malaria after three to five days' treatment, that parasites, though perhaps absent from the peripheral blood at death, are found most frequently in the spleen and marrow after oral administration of quinine, less frequently after treatment by hypodermic injections,<sup>2</sup> and with least frequency after three or four doses of 22.5 grains intravenously. It used to be not at all uncommon to find large

<sup>1</sup> *Op. cit.*, p. 344.

<sup>2</sup> These are given in a dilution of 1:20 of normal salt solution, as first originated by Dr. W. E. Deeks.

numbers of parasites after three or four days' treatment with quinine by mouth, but if quinine has been given intravenously for two or three days in doses of 22.5 to 30 grains, no matter how heavy the infection was on admission, the parasites are always scanty in the spleen and marrow at autopsy, and not infrequently only a very few are found.

I have found no reason to believe that only quinine immune parasites seek the circulation of the spleen and marrow. I hold that such parasites as are found there are those which by reason of this locality have escaped the full effects of the drug. These, and other considerations presented elsewhere in this paper, have led me to present the following hypothesis as an explanation of the fact that certain asexual parasites survive the action of quinine, and later take up a cycle of increased reproduction.

In common with others who have worked on malaria, I believe that the circulation of the bone marrow and spleen is the favored location of the *Plasmodia* in the human host. In latent malaria, when the infection is very scanty, the bone marrow and spleen are the only places where parasites are found, notwithstanding, as I have noted, the relative infrequency of erythrocytes in smears as compared with smears of the peripheral blood, and here, in small numbers, the parasites go through a normal asexual cycle. Here also, in estivo-autumnal malaria is where the gametes are formed, particularly in the bone marrow, as Marchiafava and Bignami first noted. When the parasites have multiplied beyond a certain extent they find their way into the peripheral blood, though what may be the conditions that cause this increase as manifested in febrile relapse I cannot explain.

By reason of this location, the parasites that inhabit it are to a less extent affected by quinine than those in the peripheral blood, as is shown by the persistence of the former when none can be found in the external circulation. Sufficient quinine given by the mouth, except in very severe cases, will rapidly kill the parasites in the peripheral blood, but does not always get rid of those in the internal circulation, as the persistence of gametes after the time of their cycle of development demonstrates. (See Dr. Molloy's case, and others reported in any textbook on malaria.) These residual para-

sites, since they are only partly affected by quinine (probably some are not at all affected), acquire an immunity against the drug similar to that acquired by other protozoon parasites of man under like conditions. This immunity, however, seems to pertain particularly to the parasites of the spleen and marrow. As long as there is a supply of quinine in the peripheral blood, those in it are killed. It is very unusual to observe a relapse with parasites in the external circulation during efficient quinine treatment. When such occurs, there has been faulty absorption of the drug, because the parasites disappear rapidly after subsequent hypodermic or intravenous medication. Nor does the withdrawal of quinine necessitate an immediate response by the parasites to such an extent that an increase follows, which may be detected in the peripheral blood. The asexual parasites may not increase in number for long periods, or they may increase only sufficiently to give rise to symptoms of toxemia, as was shown in the consideration of chronic malaria.

It is not possible at present to determine the conditions that control the wave-like increase and decrease which take place until the infection has been eradicated or dies spontaneously. But that such increase and decrease occur has been shown, and likewise the fact that instead of periods of increase and decrease there are also periods when the number of parasites is stationary over some time, as in the case of quartan malaria cited. And, as Sir Ronald Ross has well demonstrated, it is illogical to infer that the asexual cycle cannot outlive those periods in which it is found in the peripheral blood. The entire data of chronic malaria, as noted, tell against such an inference.

Provided there is no fault of absorption in the stomach and the intestinal canal, I do not know why it is that so often quinine by the mouth is less effective than by hypodermic injection, but I do know that this is true. Dr. W. E. Deeks has suggested that, since quinine by mouth, before reaching the systemic circulation, must pass through the liver, possibly some change in its toxicity against the *Plasmodia* takes place. It is quite certain, as Ross and Thomson's case well demonstrates, that proof of retention of quinine by urinalysis is no indication of whether the drug is killing the parasites in the internal circulation. Also cinchonism, which many rely

upon as an indication of successful exhibition of the drug, is unreliable as a test of toxicity against the parasites, for cinchonism is more indicative of the patient's susceptibility to quinine than it is that the remedy is doing the work for which it was given.

The fact that of all systems of medication the intravenous method in high dilution, as first recommended by MacGilchrist,<sup>1</sup> is the most successful in freeing the circulation of the spleen and marrow of parasites, suggests that even in the process of absorption after hypodermic injections some of the effects of the drug are lost.

#### SUMMARY.

If the hypothesis be accepted that the asexual cycle alone is the cause of relapse, and under certain conditions takes on a relative immunity against quinine, an explanation is offered of all the factors concerned in the etiology of relapse, and I know of no other hypothesis which will explain satisfactorily the correlation between these factors and the data which to this time have been collected about relapse. I may summarize these factors, which were given early in this paper, in relation to the asexual cycle as follows:

1. Relapse is one of the most common factors in malarial infection; and certainly the asexual cycle is that phase of the malarial parasites found most frequently associated with the primary infection and relapse, and with one relapse and the succeeding one.

2. Relapse follows frequently the so-called spontaneous cure of malaria, because the asexual cycle in such an instance often persists, in numbers that can be detected by the "thick film" method, or slightly below this limit, in the intervals of apyrexia.

3. Infections treated insufficiently with small doses of quinine will in all probability relapse, because the parasites of the asexual cycle in the spleen and marrow are very slightly, if at all, affected thereby.

4. Relapse is less likely to occur when the infection is promptly and vigorously treated, because the older the asexual cycle, the more resistant to quinine it becomes, as shown by numerous clinical observations.

<sup>1</sup> *Quinine and Its Salts*, Cited in *Paludism*, Simla, 1911.

5. When a relapse occurs, with manifestation of parasites in the peripheral blood, during the administration of quinine by mouth in sufficient doses, faulty absorption of the drug is sure to exist, and the method of medication should be changed.

6. The asexual generation does not have an unlimited potential of vitality. That is why, if death does not supervene in the course of a malarial infection, the infection itself will in time die out, but often not until it has done irreparable damage.

7. It is easier to eradicate an infection in persons in good health, because in these the natural protective forces of the body aid the action of quinine.

The hypothesis also concurs with the two factors in the life-cycle of the parasites, which are intimately connected with the etiology of relapse: because (*a*) quinine given by the mouth very often does not eradicate the asexual cycle in the marrow and spleen, and the residual parasites become immune; and (*b*) the longer the asexual cycle persists, the easier it acquires immunity against the drug.

The practical importance of the hypothesis lies in the value it has as a guide to treatment of malaria. Small doses of quinine, even in the mildest infections, serve only to render the asexual cycle relatively immune, so that larger doses, which, if they had been given early in the attack might have eradicated the parasites, are later without effect.

We have found this to be true by our clinical experience here in Ancon Hospital. In the treatment of malaria in our American employees, it was observed by Dr. Deeks that when the liquid preparation of quinine sulfate which we use was given in doses of 20 grains per day by mouth, while acute attacks were often controlled, recurrent cases in large numbers followed at very short intervals later, and in the treatment of these, larger doses were necessary. A routine treatment of 20 grains on diagnosis, and 10 grains three times a day later, for at least 10 days, was then established, and this we used for several years. This noticeably diminished the number of recurrent cases (we can determine relapses positively in individual cases, only under a few conditions, such as recurrence while in hospital) among the Americans, but not suffi-



ciently among the more poorly nourished European laborers. Dr. Deeks then instituted a treatment of 45 grains per day, in doses of 15 grains each. This method has practically eradicated recurrent malaria among the Americans, and to a large extent among the European laborers. These latter, however, treat mild infections themselves with small doses of quinine, and so produce a relatively quinine immune asexual cycle that is more difficult to eradicate. They are, moreover, heavily infected with syphilis, a condition which, as noted, tends to lessen the efficacy of the usual treatment until the syphilis itself is under control.

It should be stated that in the past six years our malaria rate has fallen to about one-fifth of the maximum, so that reinfections are proportionately less infrequent, but the rate was never relatively very high among the Americans except in 1905 and 1906, so that later in this class, recurrences at short intervals, which were plentiful enough, were nearly always due to relapse.

It will, of course, be obvious to anyone who is familiar with the recent work of Ross and Thomson, and of Bignami, on the etiology of relapse in malaria, that the hypothesis I have brought forward is a combination of their views, and is not altogether original with me. I desire to take this opportunity to acknowledge my indebtedness to these authorities for the use I have made of the material they have furnished me.

I wish also to thank those authorities throughout the world who sent answers to the letter of inquiry on this subject, which was addressed to them by the Department of Sanitation of the Isthmian Canal Commission. In the complete report on this subject, which will be made later by Dr. Deeks and myself, full acknowledgment of all the information received will be made.

I am indebted to many of the physicians on the medical side of Ancon Hospital for the opportunity to study cases as they occurred, particularly to Dr. Roland Connor for furnishing me with material to study quartan cases.

And I wish to thank Dr. S. T. Darling, chief of Board of Health laboratory here, and Dr. H. C. Clark, pathologist, for furnishing me with material to study from autopsy cases.

Also I thank Col. John L. Phillips, Medical Corps, U.S. Army, acting chief sanitary officer, for permission to publish this paper.

#### EXPLANATION OF PLATE 1.

These figures are intended to show, according to my interpretation, some of the phases of the *Plasmodia* which are held by others to be various stages in parthenogenesis or in conjugation.

They are drawn to the scale of  $7\mu$ , or the diameter of a normal erythrocyte, to one-fourth of an inch. The measurements were taken with a Leitz Filar micrometer,

after computing its value by use of an object micrometer. I found this method more satisfactory than the use of a camera lucida. The magnification is about 800.

FIG. 1.—Multiple infection of an erythrocyte with three young estivo-autumnal parasites. Note the overlapping of two of the parasites.

FIGS. 1a-32.—These were taken from a quartan infection that had persisted for six weeks under observation. There was a hospital record of two previous admissions for quartan infection within the previous eight months. There was marked anemia at the time when the drawings were made.

FIGS. 1a-7, 11, 12, 23, 24.—Normal schizogony of the quartan parasite. The other figures show various atypical developmental and segmentation phases. Figs. 9 and 10 particularly show part of the chromatin undivided, the so-called *Restkörper*, with the rest in collected masses prior to segmentation. All phases between this and normal segmentation may be followed in the figures. Fig. 19 shows a very large quartan parasite in irregular division, as compared with Fig. 7. In Figs. 25-32 inclusive the segmentation is very atypical.

FIGS. 33-38.—Conjugation in stained specimens, as described by Craig. Note the division of the chromatin, and the extrusion of part of it in Fig. 36.

FIGS. 39-45.—Drawn from the same specimen. These show various phases from the youngest trophozoite to beginning presegmentation. It can be seen that the nuclear phenomena, as represented by division and arrangement of the chromatin, are the same in these single parasites as in those represented as conjugating.

FIGS. 46-48.—These show double infection of the cell, because the parasites are nearly 24 hours old, as can be told by the formation of the "signets," while conjugation takes place only between very young parasites. Yet at the junction of the parasites, there is no line of demarkation, which is also true in conjugating forms.

In the infection from which Figs. 33-48 were taken, every phase of normal schizogony could be followed in the peripheral blood at the same time. Notwithstanding the large number of parasites arranged as in Figs. 33-38 inclusive (conjugation forms) all stages of further development to segmentation were typical, even in many of the doubly infected cells; in some of which latter two fully developed segmenting forms could easily be made out, while others contained two parasites at different stages of growth.

Parasites very similar to those in Figs. 9-32 inclusive are pictured by W. S. Harrison for the tertian parasite, in the *Journal of the Royal Army Medical Corps*, London, Vol. 13, No. 6 (December, 1909). In a forthcoming publication by Dr. David Thomson and myself, Dr. Thomson has drawn the phases of crescent formation referred to in the preceding article, and these will show the difference between the asexual cycle and the variations in type in the sexual cycle.

## THE INHIBITIVE ACTION OF BILE UPON *B. COLI*.\*

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Media containing bile or bile salts have come to be widely used for the isolation of *B. coli* from sewage and polluted water. Bile media have also been used extensively for the presumptive test. In the second edition of *Standard Methods of Water Analysis*,<sup>1</sup> lactose bile medium is recommended for the quantitative estimation of the *B. coli* group.

There is no doubt that the use of a bile medium offers certain advantages. Gas formation in a lactose bile fermentation tube is more likely to denote the presence of *B. coli* than gas formation in a dextrose broth fermentation tube. It is also true that in comparative series, lactose bile tubes are not so likely to show gas formation as plain lactose broth. These facts lessen the labor of plating as well as the thankless task of establishing the relation of gas-producing organisms to the *B. coli* group.

On the other side is a disadvantage not so generally recognized. This is the circumstance that *B. coli* itself is inhibited in a marked degree in media containing bile. It is true that Prescott and Winslow,<sup>2</sup> Longley and Baton,<sup>3</sup> and others have found that with some waters dextrose broth yields a larger proportion of positive results than lactose bile, but the use of different carbohydrates, lactose in one medium, dextrose in another, introduces a disturbing factor into such comparisons, and furthermore, the number of *B. coli* actually present in the water tested was an unknown factor. Ruediger,<sup>4</sup> working with polluted river water in North Dakota, has also noted inhibition in bile media as appears from the statement:

"It seems that less *B. coli* colonies will be found in the bile agar than in ordinary litmus lactose agar, although our work is not far enough advanced to draw positive conclusions."

\* Received for publication April 11, 1913.

<sup>1</sup> American Public Health Association, 1912.

<sup>2</sup> *Rept. Am. Pub. Health Assn.*, 1907, 33(2), p. 128.

<sup>3</sup> *Jour. Infect. Dis.*, 1907, 4, p. 3.

<sup>4</sup> *Jour. Am. Pub. Health Assn.*, 1911, 1, p. 831.

The extent to which bile inhibits pure cultures of *B. coli* seems not to have been determined. With a view to ascertaining the degree of inhibition, a number of pure cultures of various ages and histories were plated in suitable suspensions and in parallel series upon plain agar and bile agar.<sup>1</sup> The majority of these were saccharose-fermenting strains. The bile agar was prepared as follows:

## LACTOSE BILE AGAR

1,000 c.c. fresh, neutral ox bile  
10 gms. lactose  
10 gms. Witte's peptone  
15 gms. agar

The agar, peptone, and lactose are dissolved in bile without water, boiling as little as possible. When dissolved, the medium is filtered without titration, tubed, and sterilized in the autoclav 3 minutes at 15 pounds pressure.

The following table shows certain typical results.

TABLE I.  
COLONY COUNT  
48 Hours at 37° C.

Strain	Plain Agar	Bile Agar	
Fecal strains transferred on nutrient agar at 48-hr. intervals	<div><div>1 ( 47th transfer).....</div><div>2 (204th " ).....</div><div>3 (208th " ).....</div><div>4* ( 47th " ).....</div><div>5† ( 47th " ).....</div></div>	<div><div>390</div><div>451+</div><div>403</div><div>450</div><div>600</div></div>	<div><div>126</div><div>314</div><div>34</div><div>120</div><div>180</div></div>
Suspensions in flasks of sterile tap water‡	<div><div>6.....</div><div>7.....</div><div>8.....</div><div>9.....</div></div>	<div><div>141</div><div>683</div><div>22+</div><div>268</div></div>	<div><div>128</div><div>507</div><div>8</div><div>125</div></div>
Freshly isolated from urine and feces of different persons	<div><div>10.....</div><div>11.....</div><div>12.....</div><div>13.....</div><div>14.....</div></div>	<div><div>125</div><div>263</div><div>226</div><div>341</div><div>820</div></div>	<div><div>40</div><div>28</div><div>25</div><div>85</div><div>22</div></div>

\* Culture incubated for 2 weeks at 37° C. before plating.

† Culture kept 2 weeks in ice-chest before plating.

‡ Water suspension of pure culture kept 1 year at room temperature.

It is thus apparent that both freshly isolated strains of *B. coli* and those under long cultivation are inhibited by bile to a noteworthy degree. Four strains suspended in flasks of tap water in the laboratory at room temperature for one year showed at least as large a proportion of bile-viable cells as did the same strains grown on agar with 2-day transfers during the same period. Several strains freshly isolated from human feces were inhibited to a some-

<sup>1</sup> I have been assisted in this work by Mr. C. C. Hommon and Miss Edith Prindeville.

what greater degree on the average than other strains grown for several scores of generations on nutrient agar. It was noticed from time to time that different strains did not behave exactly alike in their resistance to bile and that different lots of ox bile varied in restraining power.

In the use of bile media in sanitary water analysis, it has been assumed, first, that the inhibitory effect of bile upon *B. coli* was slight, second, that the *B. coli* cells that are unable to grow in bile media are those that have been "attenuated" by a long sojourn in water and are hence of little significance. The following sentences from the second edition of *Standard Methods of Water Analysis* deal with this question:

"Attenuated *B. coli* does not represent recent contamination and all *B. coli* not attenuated grows readily in lactose bile" (p. 87).

"After numerous experiments it has been found that the lactose bile medium is slightly inhibitive to *B. coli* especially in attenuated form so that any positive tests with this medium indicate recent or fresh contamination" (p. 88).

"In the interpretation of the sanitary quality of the water, it is best to discount the presence of attenuated *B. coli* and to be sure to obtain all vigorous types. The lactose bile medium accomplishes both of these objects" (p. 91).

The belief that inability to grow in bile media is increased by prolonged suspension in water seems to be contradicted by the observations recorded above. Whether the inhibition shown in Table 1 is slight, is a matter of opinion.

With a view to testing the question of "attenuation" still further, 100 colonies of *B. coli* were fished from pure culture plates on plain agar and the same number from bile agar plates. The conditions were made absolutely parallel in all cases. If it were true that the more "vigorous" cells grew only on bile agar, one might, perhaps, expect more vigorous manifestations of physiological activity from the bile agar colonies than from those off the plain agar. Tested for milk coagulation (48 hours) and maximum indol production (4 days), the following results were obtained:

	Total Number of <i>B. coli</i> Fished	Milk Coagulation (48 Hours)	Maximum Indol Pro- duction (4 Days)
Plain agar.....	100	75	41
Bile agar.....	100	73	37

So far as these observations go, therefore, the cells that grow on bile agar are no more "vigorous" as regards fermentative and proteolytic power than the cells growing on plain agar. It is possible to assume of course that some of the cells growing on bile agar have been affected by the inhibiting agent in such a way that they have been reduced in their biological qualities to the level of the "weaker" cells growing on plain agar, but such an assumption puts the question beyond the range of experimentation.

It is impossible for obvious reasons to apply to methods of water examination the results obtained with pure cultures without further testing. Bile media have been used in water analysis in two somewhat different ways: (1) as "enrichment" media where the water is incubated, for example, in lactose bile and plated out after 24 to 48 hours' growth; this preliminary step is followed by definite identification of *B. coli*; (2) as "presumptive tests" where a given amount of gas (25 per cent after 72 hours' incubation—Jackson) is considered to be positive evidence of the presence of *B. coli*. Two questions, related but distinct, arise in this connection: first, to what extent we are justified in assuming that *B. coli* is present in lactose bile tubes yielding more than 25 per cent of gas; second, whether the *B. coli* cells surviving incubation in bile media—either in "preliminary enrichment" or in "presumptive tests"—fairly represent the number of colon bacilli originally present in the water.

There is little if any doubt that nearly all lactose bile tubes, giving more than 25 per cent of gas after 72 hours, contain *B. coli*, as has been shown by Jackson,<sup>1</sup> Prescott and Winslow,<sup>2</sup> and others. Frost<sup>3</sup> found that *B. coli* could be demonstrated in over 90 per cent of lactose bile tubes showing 20 per cent or more of gas. My own experience (Tables 3 and 4) practically coincides with that of Frost. This simply means, however, that other gas-producing bacteria are inhibited in larger measure than *B. coli* and indicates nothing as to the extent to which *B. coli* cells themselves refuse to grow in lactose bile. Some tests were made therefore with samples of water and sewage, unusual pains being taken to isolate *B. coli* from

<sup>1</sup> *Jour. Infect. Dis.*, Suppl. No. 3, 1907, p. 33.

<sup>2</sup> *Rept. Am. Pub. Health Assn.*, 1907, 33 (2) p. 128.

<sup>3</sup> *Hyg. Lab. Bull.*, No. 78, p. 134. Washington, 1911.

every sample giving the standard indications. In the observations here set down, ox bile freshly obtained from the Union Stockyards in Chicago has been used. The media have been prepared in accordance with the second edition of *Standard Methods of Water Analysis* (Am. Pub. Health Assn., 1912).

The isolation of *B. coli* from fermentation tubes was carried out by plating in litmus lactose agar from those tubes showing gas production after 24 hours' growth at 37° C. In case typical red colonies did not appear on the plate, a second plating was made after 48 hours, and a second negative result was followed up in most cases by a third plating after 72 hours. Nearly one-fourth of the tubes in which the plating gave negative results after 24 hours gave positive results by the 48 hour plating, but a third plating succeeding two negative results was rarely successful. Colonies were fished from the litmus lactose agar plates to Russell's medium,<sup>1</sup> and from this to gelatin. It is advisable to transfer several colonies from the agar plates even when the appearance of the plate is not encouraging, since, as is well-known, *B. coli* does not always produce typical colonies on litmus lactose agar. I have isolated *B. coli* a number of times from unpromising-looking plates. Nearly all cultures giving a characteristic reaction in Russell's medium turn out to be members of the *B. coli* group, but I have always controlled the results with gelatin-tube inoculation (14 days). About two-thirds of the cultures were also inoculated into milk, but since this procedure in no case modified the conclusions drawn from the behavior of the cultures in Russell's medium and in gelatin it was discontinued.

The following tables (Tables 2 and 3) show the results obtained with Lake Michigan water as drawn from the laboratory tap (1912). In all cases compared, the same sample of water was tested simultaneously in equal amounts in the two media.

The results set forth in these tables show what has been observed with some other slightly polluted waters, namely, a larger number of positive coli identifications with lactose broth than with the bile medium. In examining such waters as the one here dealt with, however, it has been argued that only those *B. coli* cells that have

<sup>1</sup> *Jour. Med. Research*, 1912, 20, p. 217.

been in the water a long time are unable to grow in bile media and that the bile-resistant cells indicate "recent or fresh contamination." If this is the case, fresh sewage should contain a relatively

TABLE 2.  
LAKE MICHIGAN WATER (LABORATORY TAP).  
5 c.c. Samples.

AMOUNT OF GAS IN FERMENTATION TUBES	IN LACTOSE BROTH			IN LACTOSE BILE BROTH		
	No. of Samples	No. Showing <i>B. coli</i>	Percentage	No. of Samples	No. Showing <i>B. coli</i>	Percent- age
No gas . . . . .	16* (15)	0	0	24	0	0
Less than 10 per cent	0* (1)	0 (1)	0 (100)	0	0	0
10-20 per cent . . . . .	5	4	80	3	1	33
Over 20 per cent . . . . .	19	12	63	13	11	85
Total . . . . .	40	16 (17)	40 (42)	40	12	30

\* In one case a fermentation tube showing no gas in 48 hours showed 5 per cent of gas in 72 hours and *B. coli* was later isolated from this tube. The change in figures caused by this is indicated by the figures in parentheses underneath the regular 48-hour records.

The summary was made from 48-hour records of the lactose broth tubes and from 72-hours records of the lactose bile tubes.

In this series, tubes of liver broth (Jackson and Muer, *Jour. Infect. Dis.*, 1911, 8, p. 289) were also inoculated (40 samples). The percentage yielding *B. coli* was about the same as with lactose broth, viz., 37.

TABLE 3.  
LAKE MICHIGAN WATER (LABORATORY TAP).  
1 c.c. Sample.

AMOUNT OF GAS IN FERMENTATION TUBES	IN LACTOSE BROTH			IN LACTOSE BILE BROTH		
	No. of Samples	No. Showing <i>B. coli</i>	Percentage	No. of Samples	No. Showing <i>B. coli</i>	Percent- age
No gas . . . . .	72	0	0	100	0	0
Less than 10 per cent	23*	6*	26	6	1	16
10-20 per cent . . . . .	17	7	41	9	3	33
Over 20 per cent . . . . .	38	33	87	35	29	83
Total . . . . .	150	46	31	150	33	22

\* In one experiment, a lactose fermentation tube containing 5 per cent of gas in 24 hours was broken so that the 48-hour gas production could not be determined. A plate had been poured before the accident and *B. coli* was later isolated from this plate.

A parallel series of 90 tubes of lactose broth and the same number of liver broth tubes gave 32 per cent of *B. coli* isolations for the former, 27 per cent for the latter.

The summary is based on records taken after lactose fermentation tubes had incubated 48 hours and bile tubes 72 hours or longer.

small proportion of *B. coli* cells refusing to grow in bile media. The following table (Table 4) gives the results obtained by examination of 70 1:100,000 c.c. samples of fresh sewage and shows that



here also lactose bile fails to indicate the presence of *B. coli* under conditions where the cells are presumably recently derived from the human body and therefore indicative of recent contamination.

TABLE 4.  
SEWAGE FROM THIRTY-NINTH ST. PUMPING STATION, CHICAGO.

AMOUNT OF GAS IN FERMENTATION TUBES	IN LACTOSE BROTH			IN LACTOSE BILE		
	No. of Samples	No. Showing <i>B. coli</i>	Percentage	No. of Samples	No. Showing <i>B. coli</i>	Percent- age
No gas.....	22	0	0	52	0	0
Less than 10 per cent.....	3	0	0	0	0	0
10-20 per cent.....	4	1	25	1	1	100
Over 20 per cent.....	41	34	83	17	15	88
Total.....	70	35	50	70	16	23

In all cases recorded above, 1 c.c. of a 1:100,000 dilution of the sewage was inoculated into each fermentation tube. An equal number of lactose broth and lactose bile tubes were made of each sample of sewage. The summary was made on the basis of 48-hour records of lactose broth and 72-hour records of lactose bile.

A parallel series of 20 tubes of lactose broth and liver broth gave 35 per cent of coli isolation for each medium.

It cannot of course be assumed out of hand that lactose broth reveals the presence of all viable cells of *B. coli*. I have elsewhere called attention<sup>1</sup> to the advantage of direct plating on Endo medium for rapid isolation of *B. coli* from certain kinds of water. In the course of this work, it was found that as many colon bacilli grew from pure cultures plated on Endo medium as on plain agar.<sup>2</sup>

TABLE 5.  
COLONY COUNT (24 HOURS AT 37° C.).

Strain	Plain Agar	Endo Medium†
Fecal strains transferred on nutrient agar at 48-hr. intervals { 1 (208th transfer).....	346	363
{ 2* (11th " ".....	19	20
Suspension in flasks of sterile tap water‡ { 3.....	24	20+
{ 4.....	267	324
{ 5.....	137	156
Freshly isolated from feces 6.....	897	735

\* Water suspension kept 8½ days at room temperature before plating.

† Water suspensions kept 1 year at room temperature before plating.

These results seem within the range of counts often obtained with duplicate samples.

‡ The Endo medium used in this comparison was prepared in the following way: 1,000 c.c. sugar-free broth to which 1 per cent Witte's peptone and 20 gms. of agar have been added. Dissolve in autoclav. Make 0.5 per cent acid with sodium carbonate. Add 10 gms. of lactose and dissolve. Then add 7 c.c. of a saturated alcoholic solution of fuchsin. Decolorize with 16-25 c.c. of a 10 per cent solution of sodium sulfite. A thin plate of the medium, when cool, should be a very pale pink. When sufficiently decolorized, filter, tube, and sterilize for 3 min. at 15 pounds pressure in the autoclav.

<sup>1</sup> E. O. Jordan, "The Bacterial Examination of Water", *Proc. 15, International Congress of Hygiene*, 1912.

<sup>2</sup> Kinyoun's experience seems to have been different from this, since the Endo medium prepared by him inhibited colon bacilli (*Am. Jour. Pub. Health*, 1912, 2, p. 979).

If, as these results indicate, the Endo medium that I have used exerts little or no inhibitive power upon colon bacilli, a comparison of the results with this medium and with lactose broth should be of interest. I have made a few observations in this direction, but a more extended series would perhaps be desirable. The results as far as they go show equal efficiency in the two methods (Table 6). Table 7 shows in another way the suppression of *B. coli* cells by bile. Suspension of pure cultures of *B. coli* in flasks of sterilized water were inoculated in appropriate dilution and in strictly parallel series into the ordinary lactose broth and lactose bile fermentation tubes.

TABLE 6.  
PROPORTION OF *B. coli* ISOLATED FROM LACTOSE BROTH AND ENDO PLATES.

TOTAL QUANTITY EXAMINED	NO. OF <i>B. coli</i> ISOLATED FROM	
	Lactose Broth	Endo Plates
100 c.c. (Lake Michigan water)*.....	39	39
5-10,000 c.c. (sewage)†.....	28	32

\* A summary of 10 experiments in each of which 10 c.c. of the sample was plated on Endo's medium and 10 c.c. inoculated into lactose broth fermentation tubes. All colonies at all resembling *B. coli* were picked from the Endo plates, and all gas-producing tubes of lactose broth were plated out in the manner above described (p. 0007).

† A summary of 5 experiments, in each of which 1-10,000 c.c. of the sample was plated on Endo's medium and 1-100,000 c.c. inoculated into each of 10 lactose broth fermentation tubes, making the total amount of the sample the same in both cases.

TABLE 7.  
WATER SUSPENSIONS\* OF PURE CULTURES OF *B. coli* IN LACTOSE BILE AND LACTOSE BROTH.

Amount of Gas in Fermentation Tube	In Lactose Broth†	In Lactose Bile†
No gas.....	12	22
More than 20 per cent.....	18	8
Total number of samples.....	30	30

† Readings made after 48 hours at 37° C.

\* Kept for 102 days at room temperature.

The results as given in the table show about the same degree of inhibition as appears in the experiment with sewage (Table 4).

These observations show that bile inhibits at least from one-third to one-half of the viable cells of *B. coli* and sometimes a much larger proportion; that freshly isolated cultures are inhibited in at least the same degree as those under long cultivation or those

subjected to prolonged sojourn in water; that there is no evidence that *B. coli* cells that are unable to grow on bile medium are any more "attenuated" or less "vigorous," biologically, than their fellows, and that with some care in making dilutions and replating if necessary *B. coli* will be isolated in a larger proportion of cases from lactose broth fermentation tubes than from lactose bile. These facts may not modify the advantages claimed for bile media for presumptive tests, but they do demonstrate that bile is an inhibiting substance for *B. coli* as for other microorganisms and that its use always involves the suppression of a certain number of viable cells. The cells that are suppressed cannot be assumed to be any less significant in the interpretation of sanitary water analyses than the cells actually surviving the passage through bile.

## THE USE OF VIRULENT SALT SOLUTION AS A VIRUS IN MANUFACTURING HOG CHOLERA SERUM.\*

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Soon after the demonstration at Ames, Iowa, in 1908, to show methods of manufacture and value of Dorset-Niles serum toward controlling hog cholera, many of the agricultural experiment stations became interested and have since put forth vigorous efforts toward controlling this disease with hog cholera serum.

Although carrying many unsolved problems, hyperimmune serum has been efficient in preventing cholera and no doubt has been of great aid in controlling this disease. The control of cholera in any section depends to a great extent upon the expense involved, and, in case the serum is to be used, its price must necessarily be considered. Therefore, considering the cost of producing serum, there seems to be a demand for research which will result in a reduction of the expense.

In manufacturing serum by the regular Dorset-Niles methods, the cholera pig is sacrificed merely for its blood, and this virus blood, unless injected by the intravenous method, is sufficient only to hyperimmunize one pig of weight equal to that of the virus pig. Thus, we can readily see that reducing the cost of manufacturing serum by this method depends to a great extent upon reducing the cost of a virus that will prove efficient in hyperimmunizing.

Taking up the idea of Dr. Craig, of the Indiana Agricultural Experiment Station, this experiment was undertaken as a problem of practical value, with the purpose of reducing the cost of serum by using salt solution as a virus when passed through the abdominal cavity of virus pigs. It was our desire to determine the value of this saline solution as a virus and, if possible, to advance methods of using it that would prove efficient and practical.

Since an accurate method of standardizing serum or virus is

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lacking, and the virulence of virus varies according to its source, and the potency of serum varies according to virulence of virus used, amount of virus injected, and condition of pig used for hyper-immune before and after injecting, I found that it required numerous and careful tests to establish our results, each being run parallel with the Dorset-Niles subcutaneous methods as a check.

In this experiment, I used 0.75 per cent to 0.85 per cent salt solution. The solution was sterilized and kept so in cotton-plugged flasks. Just before using, it was heated to about 37.5° C. It was injected at this temperature by means of a sterile aspirator apparatus into the abdominal cavities of virus pigs, in varying amounts, and allowed to remain various lengths of time so that we might determine just what time would prove most satisfactory and the approximate amount to inject. That portion of the solution remaining unabsorbed was removed from the peritoneal cavity by means of a sterile pipette or a small casserole immediately after killing the pig.

In this work, I have injected 56 virus pigs with salt solution. The injection varied in amount from 20 c.c. to 45 c.c. per pound of body weight and varied in time remaining in abdominal cavity from 3 hours to 14 hours as shown in Table 1 of virus pigs.

• With the virulent salt solution secured from abdominal cavities of virus pigs, I have hyperimmunized 43 pigs, injecting the virus subcutaneously at the rate of from 10 c.c. to 20 c.c. per pound of body weight as shown in the following table of hyperimmunes (Table 2).

It is generally admitted by serum manufacturers that the amount of virus blood secured from virus pigs averages about 10 c.c. per pound of body weight. Accepting this as a standard, I have endeavored to calculate the percentage of increase of virulent material secured when salt solution was injected intra-abdominally as follows (Table 3).

How does salt solution gain virulence when injected into abdominal cavity of virus pigs? It is impossible at present to state just how salt solution gains its virulence while in the abdominal cavity, and knowing very little of the ultramicroscopic virus causing hog cholera, it is impossible to determine just how virulent

this solution may become. The virulence no doubt varies greatly with different pigs even though autopsies correspond. As far as

TABLE 1.

EXPERIMENTAL VIRUS PIGS INJECTED INTRA-ABDOMINALLY WITH PHYSIOLOGICAL SALT SOLUTION.

Pig No.	Wt. Lbs.	Amount of NaCl Solution Injected	No. c.c. per Lb.	Time Remaining in Abdominal Cavity	Amount of Virus NaCl Recovered	Percentage Recovered	Average Percentage Recovered	Amount of Virus Blood Secured	Approximate Increase of Virus Blood Due to NaCl Injected
267.....	79	2,000 c.c.	25	3 hrs.	1,250 c.c.	62.5	....	875 c.c.	85 c.c.
264.....	110	2,750 c.c.	25	3 hrs.	1,075 c.c.	71.8	67.1	1,250 c.c.	150 c.c.
270.....	121	3,025 c.c.	25	4 hrs.	1,500 c.c.	49.5	....	1,550 c.c.	340 c.c.
272.....	81	2,025 c.c.	25	4 hrs.	1,550 c.c.	76.5	....	1,550 c.c.	740 c.c.
276.....	100	2,500 c.c.	25	4 hrs.	1,550 c.c.	62.0	....	1,400 c.c.	400 c.c.
278.....	78	1,950 c.c.	25	4 hrs.	1,100 c.c.	56.4	....	1,000 c.c.	320 c.c.
277.....	122	3,000 c.c.	25	4 hrs.	1,300 c.c.	43.3	....	850 c.c.	.....
461.....	71	1,875 c.c.	25	4 hrs.	1,000 c.c.	53.3	56.8	900 c.c.	190 c.c.
433.....	80	1,600 c.c.	20	4 hrs.	650 c.c.	40.6	40.6	1,100 c.c.	300 c.c.
284.....	94	2,820 c.c.	30	4 hrs.	1,400 c.c.	49.6	....	1,200 c.c.	260 c.c.
479.....	68	2,040 c.c.	30	4 hrs.	700 c.c.	34.3	41.9	600 c.c.	.....
476.....	90	4,050 c.c.	45	4 hrs.	2,200 c.c.	54.8	54.8	1,100 c.c.	200 c.c.
280.....	82	2,460 c.c.	30	5 hrs.	1,500 c.c.	65.0	....	1,100 c.c.	280 c.c.
281.....	134	4,020 c.c.	30	5 hrs.	2,600 c.c.	67.1	....	1,600 c.c.	260 c.c.
282.....	124	3,750 c.c.	30	5 hrs.	2,200 c.c.	58.6	....	1,000 c.c.	.....
283.....	108	3,300 c.c.	30	5 hrs.	2,600 c.c.	78.7	....	1,650 c.c.	560 c.c.
285.....	128	3,840 c.c.	30	5 hrs.	2,200 c.c.	57.0	....	1,650 c.c.	360 c.c.
290.....	95	2,700 c.c.	30	5 hrs.	1,400 c.c.	51.1	....	1,350 c.c.	400 c.c.
291.....	96	2,880 c.c.	30	5 hrs.	2,000 c.c.	62.5	....	1,100 c.c.	140 c.c.
292.....	83	2,490 c.c.	30	5 hrs.	1,250 c.c.	52.2	....	1,200 c.c.	270 c.c.
422.....	100	3,000 c.c.	30	5 hrs.	1,800 c.c.	60.0	....	1,100 c.c.	100 c.c.
474.....	76	2,300 c.c.	30	5 hrs.	600 c.c.	26.0	57.8	1,000 c.c.	240 c.c.
434.....	88	3,000 c.c.	35	5 hrs.	2,000 c.c.	66.6	....	1,100 c.c.	220 c.c.
493.....	90	3,250 c.c.	35	5 hrs.	1,500 c.c.	46.1	46.1	1,200 c.c.	300 c.c.
443.....	75	3,000 c.c.	40	5 hrs.	1,200 c.c.	40.0	....	1,100 c.c.	350 c.c.
454.....	53	2,250 c.c.	40	5 hrs.	1,400 c.c.	63.0	51.5	1,200 c.c.	670 c.c.
466.....	60	2,700 c.c.	45	5 hrs.	1,300 c.c.	48.1	....	1,100 c.c.	500 c.c.
485.....	100	4,500 c.c.	45	5 hrs.	2,700 c.c.	60.0	54.5	1,400 c.c.	400 c.c.
403.....	117	3,000 c.c.	25	6 hrs.	2,000 c.c.	66.6	....	1,600 c.c.	430 c.c.
384.....	88	3,000 c.c.	30	6 hrs.	2,000 c.c.	66.6	....	1,100 c.c.	220 c.c.
431.....	100	3,000 c.c.	30	6 hrs.	1,000 c.c.	33.4	50.0	1,700 c.c.	700 c.c.
341.....	85	2,500 c.c.	33	6 hrs.	900 c.c.	36.0	....	1,000 c.c.	150 c.c.
414.....	85	3,000 c.c.	36	6 hrs.	1,600 c.c.	53.3	....	1,000 c.c.	150 c.c.
432.....	72	2,500 c.c.	36	6 hrs.	1,200 c.c.	48.0	50.5	1,000 c.c.	280 c.c.
438.....	73	3,000 c.c.	40	6 hrs.	2,000 c.c.	66.6	....	1,400 c.c.	670 c.c.
423d.....	77	3,200 c.c.	40	6 hrs.	1,700 c.c.	53.1	59.5	1,000 c.c.	230 c.c.
422b.....	60	2,500 c.c.	42	6 hrs.	1,400 c.c.	56.0	56.0	1,100 c.c.	500 c.c.
461b.....	70	3,150 c.c.	45	6 hrs.	1,200 c.c.	38.0	38.0	1,220 c.c.	420 c.c.
415.....	104	3,000 c.c.	27	6½ hrs.	2,000 c.c.	66.6	66.6	1,700 c.c.	660 c.c.
407.....	107	2,500 c.c.	23	7 hrs.	1,000 c.c.	40.0	40.0	1,200 c.c.	130 c.c.
286.....	96	3,000 c.c.	30	7 hrs.	1,800 c.c.	60.0	....	1,200 c.c.	240 c.c.
418a.....	88	2,700 c.c.	30	7 hrs.	650 c.c.	24.0	42.0	1,200 c.c.	320 c.c.
423b.....	93	3,000 c.c.	31	7 hrs.	1,200 c.c.	40.0	40.0	1,200 c.c.	270 c.c.
346.....	100	3,400 c.c.	34	7 hrs.	1,800 c.c.	52.9	52.9	1,600 c.c.	600 c.c.
435.....	78	3,000 c.c.	38	7 hrs.	1,300 c.c.	43.3	43.3	1,000 c.c.	220 c.c.
431.....	70½	3,000 c.c.	42	7 hrs.	2,100 c.c.	70.0	70.0	1,700 c.c.	1,000 c.c.
404.....	143	3,000 c.c.	20	7½ hrs.	2,000 c.c.	66.6	66.6	2,400 c.c.	970 c.c.
406.....	103	2,500 c.c.	24	7½ hrs.	1,000 c.c.	40.0	40.0	1,100 c.c.	70 c.c.
289.....	98	3,000 c.c.	30	7½ hrs.	2,100 c.c.	70.0	70.0	1,200 c.c.	220 c.c.
380.....	77	3,000 c.c.	39	7½ hrs.	2,200 c.c.	73.3	73.3	1,200 c.c.	430 c.c.
428.....	68	2,680 c.c.	40	7½ hrs.	2,000 c.c.	74.6	74.6	1,000 c.c.	320 c.c.
402.....	132	3,000 c.c.	22	8 hrs.	2,000 c.c.	66.6	66.6	1,600 c.c.	280 c.c.
417.....	92	2,700 c.c.	30	8 hrs.	None	00.0	00.0	1,000 c.c.	80 c.c.
417.....	84	2,000 c.c.	24	9 hrs.	900 c.c.	45.0	45.0	700 c.c.	.....
408.....	93	2,500 c.c.	26	11 hrs.	1,200 c.c.	48.0	48.0	1,250 c.c.	320 c.c.
418b.....	88	2,000 c.c.	25	14 hrs.	None	00.0	00.0	1,200 c.c.	320 c.c.

our methods of standardizing virus go, we fail to see a decrease in virulence of virus blood due to injecting salt solution, although

TABLE 2.  
EXPERIMENTAL PIGS, HYPERIMMUNIZED WITH VIRUS SALT SOLUTION.

HYPER- IMMUNE No.	WEIGHT IN POUNDS	AMOUNT OF VIRUS NaCl INJECTED TO HYPERIMMUNIZE	No. c.c. INJECTED PER LB. OF BODY WEIGHT.	DATA ON VIRUS NaCl USED FOR HYPER- IMMUNIZATION				AMOUNT OF VIRUS NaCl INJECTED TO REHYPER- IMMUNIZE	No. c.c. PER LB. INJECTED TO REHYPERIMMUNIZE	DATA ON VIRUS NaCl USED FOR REHYPER- IMMUNIZATION				TOTAL BLEEDINGS OR HYPER- IMMUNES	TOTAL AMOUNT OF SERUM SECURED	RESULT OF SERUM TEST	
				Time Virus Remained in Abdominal Cavity of Virus Pigs	No. c.c. per Lb. of NaCl Sol. Inf. into Abdominal Cavity of Virus Pigs	Percent of NaCl Sol. Re- covered from Virus Pigs	Time Virus NaCl Re- mained in Abdominal Cavity of Virus Pigs	Percent of Injected NaCl Sol. Recovered from Virus Pigs	No. c.c. NaCl per Lb. Inf. into Abdominal Cavity of Virus Pigs							No. c.c. Protected Test Pigs against 2 c.c. Virus Blood	No. c.c. Failed to Protect Test Pigs
246.....	84	630 c.c. NaCl 420 c.c. V.B.	7½	3 hrs.	25	62.5	.....	.....	.....	.....	.....	.....	.....	4	2,700 c.c.	35	10-15-20 25-30
247.....	90	450 c.c. NaCl 450 c.c. V.B.	5	3 hrs.	25	62.5	.....	.....	.....	.....	.....	.....	.....	4	2,900 c.c.	.....	10-15-20-25 10-15-30-35
250.....	90	900 c.c. NaCl	10	3 hrs.	25	71.8	.....	.....	.....	.....	.....	.....	.....	4	3,000 c.c.	35	10-15-20 25-30
320.....	65	650 c.c. NaCl 715 c.c. V.B.	10	4 hrs.	20	40.6	.....	.....	.....	.....	.....	.....	.....	4	2,800 c.c.	20	10-15
251.....	95	475 c.c. NaCl 360 c.c. V.B.	12½	4 hrs.	25	76.5	.....	.....	.....	.....	.....	.....	.....	4	2,900 c.c.	10-15-20	.....
252.....	72	360 c.c. NaCl	5	4 hrs.	25	70.5	.....	.....	.....	.....	.....	.....	.....	4	2,200 c.c.	15-20	10
254.....	118	1,475 c.c. NaCl	12½	4 hrs.	25	40.5	.....	.....	.....	.....	.....	.....	.....	4	3,400 c.c.	10-15-20	.....
256.....	106	1,060 c.c. NaCl	10	4 hrs.	25	62.0	.....	.....	.....	.....	.....	.....	.....	4	3,300 c.c.	15-20	10
257.....	120	610 c.c. NaCl 610 c.c. V.B.	5	4 hrs.	25	56.4	.....	.....	.....	.....	.....	.....	.....	4	4,000 c.c.	10-15-20	.....
258.....	80	400 c.c. NaCl 400 c.c. V.B.	5	4 hrs.	25	56.4	.....	.....	.....	.....	.....	.....	.....	4	2,700 c.c.	10-20	15
260.....	128	1,300 c.c. NaCl	10	4 hrs.	25	43.3	.....	.....	.....	.....	.....	.....	.....	4	4,400 c.c.	10-15-20	.....
330.....	80	800 c.c. NaCl 700 c.c. V.B.	10	4 hrs.	25	53.3	.....	.....	.....	.....	.....	.....	.....	4	3,000 c.c.	20	10-15
323.....	100	300 c.c. NaCl 300 c.c. V.B.	7	4 hrs.	30	34.3	.....	.....	.....	.....	.....	.....	.....	4	2,700 c.c.	20-25	10-15
267.....	135	1,400 c.c. NaCl	10	4 hrs.	30	40.6	.....	.....	.....	.....	.....	.....	.....	4	4,500 c.c.	10-15-20	.....
341.....	110	1,100 c.c. NaCl	10	4 hrs.	45	54.8	.....	.....	.....	.....	.....	.....	.....	4	3,605 c.c.	20	10-15
269.....	105	1,575 c.c. NaCl	15	5 hrs.	30	67.1	.....	.....	.....	.....	.....	.....	.....	4	3,800 c.c.	15-20	10
268.....	135	2,025 c.c. NaCl	15	5 hrs.	30	72.9	.....	.....	.....	.....	.....	.....	.....	4	3,150 c.c.	10-15-20	.....
270.....	120	1,800 c.c. NaCl	15	5 hrs.	30	70.0	.....	.....	.....	.....	.....	.....	.....	7	4,470 c.c.	10-15-20	.....
262.....	160	2,400 c.c. NaCl	15	5 hrs.	30	57.3	.....	.....	.....	.....	.....	.....	.....	4	4,500 c.c.	10-15-20	.....
263.....	150	1,000 c.c. NaCl	15	5 hrs.	30	57.3	.....	.....	.....	.....	.....	.....	.....	4	4,600 c.c.	15-20	10

TABLE 2—Continued

290.....	120	600 c.c. NaCl	5	5 hrs.	30	52.2	.....	.....	.....	.....	.....	4	3,500 c.c.	10-15-20	.....
291.....	120	600 c.c. V.B.	5	5 hrs.	30	52.2	.....	.....	.....	.....	.....	4	3,500 c.c.	10-15-20	.....
297.....	120	600 c.c. NaCl	5	5 hrs.	30	70.2	.....	.....	.....	.....	.....	4	3,500 c.c.	10-15-20	.....
298.....	120	600 c.c. V.B.	12	5 hrs.	30	70.2	.....	.....	.....	.....	.....	4	3,500 c.c.	10-15-20	.....
322.....	90	600 c.c. NaCl	10	5 hrs.	30	26.0	.....	.....	.....	.....	.....	4	3,000 c.c.	10-15-20	.....
323.....	120	600 c.c. V.B.	10	5 hrs.	35	46.1	.....	.....	.....	.....	.....	4	3,300 c.c.	.....	.....
324.....	100	1,000 c.c. NaCl	10	5 hrs.	40	63.0	.....	.....	.....	.....	.....	4	3,200 c.c.	10-15-20	.....
334.....	90	900 c.c. NaCl	10	5 hrs.	40	40.0	.....	.....	.....	.....	.....	4	2,670 c.c.	15-20	.....
324.....	100	1,000 c.c. NaCl	10	5 hrs.	45	48.1	.....	.....	.....	.....	.....	4	2,785 c.c.	20	10-15
324.....	90	900 c.c. NaCl	10	5 hrs.	45	60.0	.....	.....	.....	.....	.....	4	3,070 c.c.	20	10-15
343.....	85	1,285 c.c. NaCl	15	5 hrs.	45	60.0	.....	.....	.....	.....	.....	4	3,085 c.c.	15-20	.....
309.....	147	2,550 c.c. NaCl	18	6 hrs.	30	50.0	.....	.....	.....	.....	.....	4	3,055 c.c.	10-15-20	.....
326.....	80	800 c.c. NaCl	10	6 hrs.	40	53.1	.....	.....	.....	.....	.....	4	2,805 c.c.	20	10-15
327.....	82	820 c.c. NaCl	10	6 hrs.	40	53.1	.....	.....	.....	.....	.....	4	3,105 c.c.	20	10-15
336.....	120	1,200 c.c. NaCl	10	6 hrs.	45	38	.....	.....	.....	.....	.....	4	4,105 c.c.	15-20	.....
277.....	225	3,650 c.c. NaCl	16	5 hrs.	30	55.4	8	7 hrs.	52.9	24	.....	7	8,715 c.c.	5-10-15	.....
282.....	232	3,350 c.c. NaCl	14½	5 hrs.	30	62.5	8	5 hrs.	50.0	30	.....	7	8,750 c.c.	10-15	5
283.....	200	3,400 c.c. NaCl	16	6 hrs.	25	62.5	8	6 hrs.	50.0	40	.....	7	9,160 c.c.	10-15-20	.....
284.....	230	3,100 c.c. NaCl	14	7½ hrs.	30	62.5	7½	6½ hrs.	66.6	27	.....	7	9,100 c.c.	10-15-20	.....
285.....	228	3,400 c.c. NaCl	14½	8 hrs.	25	66.6	7½	6 hrs.	53.3	36	.....	7	9,465 c.c.	5-10-15	.....
310.....	98	1,470 c.c. NaCl	15	5 hrs.	30	52.7	8	5 hrs.	42.0	30	.....	7	3,200 c.c.	15-20	10
303.....	170	3,000 c.c. NaCl	18	7½ hrs.	30	50.0	.....	.....	.....	.....	.....	4	8,435 c.c.	15-20	10
312.....	145	3,000 c.c. NaCl	20	6 hrs.	33	50.0	9	6 hrs.	50.0	40	.....	7	6,000 c.c.	20	10-15
308.....	152	3,000 c.c. NaCl	15	7½ hrs.	39	49.1	10	6 hrs.	68.0	40	.....	7	4,435 c.c.	20	10-15
				7½ hrs.	48	73.9	.....	.....	.....	.....	.....	4			
				7½ hrs.	38		.....	.....	.....	.....	.....				



the volume of virus blood is considerably increased by the injection of salt solution.

TABLE 3.

No. of Pigs Used	Rate of Injection per Lb.	Time Let Remain	Average Percentage Increase	Value of Virus NaCl
2.....	25 c.c.	3 hrs.	183	Produced sera of low potency when used at rate of 10 c.c.-12½ c.c. per lb. of body wt. of immune.
6.....	25 c.c.	4 hrs.	147	Produced potent sera when used at rate of 10 c.c. 12½ c.c. per lb. in hyperimmunizing.
2.....	30 c.c.	4 hrs.	142	Produced potent sera when injected at rate of 10 c.c. per lb. in hyperimmunizing.
1.....	45 c.c.	4 hrs.	266	Produced sera of low potency when used at rate of 10 c.c. per lb.
10.....	30 c.c.	5 hrs.	199	Produced very potent sera when injected at rate of 10 c.c.-12 c.c.-15 c.c. per lb. in hyperimmunizing.
2.....	40 c.c.	5 hrs.	282	Produced potent sera when used at rate of 10 c.c. per lb. in hyperimmunizing.
2.....	45 c.c.	5 hrs.	306	Produced sera of low potency when used at rate of 10 c.c. per lb. in hyperimmunizing.
1.....	25 c.c.	6 hrs.	156	Produced potent sera.
2.....	30 c.c.	6 hrs.	213	Produced very potent sera when used at rate of 18 c.c. per lb. in hyperimmunizing.
1.....	33 c.c.	6 hrs.	125	Produced potent sera.
2.....	36 c.c.	6 hrs.	205	Produced potent sera when used with other virus.
3.....	40 c.c.	6 hrs.	309	Produced sera of low potency when used at rate of 10 c.c. per lb. in hyperimmunizing.
1.....	45 c.c.	6 hrs.	231	Produced serum with fair potency when used at rate of 10 c.c. per lb.
1.....	27 c.c.	6½ hrs.	255	Produced potent sera.
3.....	30 c.c.	7 hrs.	161	Produced very potent sera when used with other virus in hyperimmunizing.
1.....	34 c.c.	7 hrs.	240	Produced potent sera.
1.....	38 c.c.	7 hrs.	196	Produced potent sera when used with other virus in hyperimmunizing.
2.....	40 c.c.	7½ hrs.	455	Produced sera of low potency when used at rate of 15 c.c. per lb. in hyperimmunizing.
1.....	22 c.c.	8 hrs.	172	Produced very potent sera when used together with other virus.
1.....	24 c.c.	9 hrs.	102	Produced potent sera when used together with other virus.
1.....	26 c.c.	11 hrs.	163	Produced very potent sera.
1.....	25 c.c.	14 hrs.	36	

## SUMMARY.

The virulence of salt solution recovered from abdominal cavity of virus pigs varies greatly with the amount of solution injected as well as with the time the solution remains in the cavity.

The percentage of injected solution recovered varies greatly with size and age of pig as well as with time it remains in the cavity.

Salt solution injected into abdominal cavity of virus pigs in amounts not exceeding 30 c.c. per pound of body weight and allowed to remain not less than five hours is efficient in hyperimmunizing pigs.

The use of salt solution as a virus greatly increases supply of virus and may prove a means of greatly reducing the cost of manufacturing serum.

# IS THE SPECIFICITY OF THE ANAPHYLAXIS REACTION DEPENDENT ON THE CHEMICAL CONSTITUTION OF THE PROTEINS OR ON THEIR BIOLOGICAL RELATIONS?\*

## THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS. II.

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In a previous paper<sup>2</sup> it was noted that zein, the alcohol-soluble protein of corn, did not cause the anaphylaxis reaction in animals sensitized with gliadin, the alcohol-soluble protein of wheat, nor with hordein, the alcohol-soluble protein of barley. At that time hordein and gliadin had not been tested against one another, but it had been observed that preparations of gliadin, from either wheat or rye, interacted against one another<sup>3</sup> as if they were one and the same protein. On testing hordein from barley against gliadin from either wheat or rye, we have since found that these two proteins of different origin also react well with one another. Furthermore, we have found that while our preparations of gliadin from wheat react anaphylactically with glutenin from the same seed, hordein from barley fails to cause reactions in guinea-pigs sensitized with glutenin. These results have raised the question whether the specificity of the anaphylaxis reaction is not, in fact, determined solely by the chemical constitution of the proteins used as antigens.

It is true that the anaphylaxis reaction has been obtained between fluids and extracts of tissues of animals or plants of different, though closely related, species, but these fluids, or extracts, have been composed of mixtures of so many substances of practically unknown chemical constitution that attention hereto-

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<sup>2</sup> A portion of the expenses of this investigation was also shared by the Carnegie Institution of Washington, D.C.

<sup>3</sup> Wells and Osborne, *Jour. Infect. Dis.*, 1911, 8, p. 66.

<sup>4</sup> For the avoidance of circumlocution the phrase "interacted with one another" is used, meaning that animals sensitized with one protein reacted when given the other, and animals sensitized with the second protein reacted when given the first. Throughout the article wherever it is stated that one protein does or does not react with another, it is to be understood that this is used as an abbreviation to indicate the above relationship.

fore has been directed almost exclusively to the biological relations of the substances causing the anaphylaxis reaction, and relatively little consideration has, as yet, been given to their possible chemical relations.

It is impossible to state how nearly related wheat and barley actually are, for the morphological characters, on which botanical classification is based, are not necessarily accurate indices of biological relationships. However closely akin wheat and barley may be, we have in our results an instance in which an isolated and purified protein constituent of the seed of one species reacts biologically with one of the isolated proteins of the seed of another species, but not with a second purified protein from the same seed, although the two proteins from the latter seed produce severe reactions when tested against one another.

We have, furthermore, found evidence of two reacting elements in gliadin and hordein, one of which is common to both proteins. Whether this means that our preparations of gliadin and hordein consist of two or more proteins, one of which is common to both the preparations which we designate gliadin and hordein, or that gliadin and hordein are single chemical individuals which contain two or more groups capable of acting as specific antigens, cannot be settled, for as yet we have no means by which the chemical individuality of any protein can be definitely established.

The physical and chemical properties of all proteins are such that none of the means employed for establishing the chemical individuality of organic compounds can be applied to them. The best that can be done at present is to subject the isolated products to extensive fractional precipitation and to determine whether, or not, differences can be detected between the various fractions thus obtained. Such examinations have been made of the proteins used in this investigation, and no evidence was secured which indicated that any of them were mixtures. The earlier view of Ritt-hausen that preparations of gliadin and hordein, as made by our methods, contain two or more different proteins was not established by the extensive fractionations made by one of us, and subsequent experience has further confirmed our view that each of these preparations contains only a single protein. Of course the character of the evidence thus obtained does not preclude the possibility that

these, like all other so-called individual proteins, are, in fact, mixtures of two or more different, but similar, proteins. Such an assumption, however, is purely speculative, and, as yet, has only a little indirect experimental evidence to support it.<sup>1</sup>

The main point of interest for us at the present time is that in the reactions described in this paper we have a definite indication that *the specificity of the anaphylaxis reaction is determined by the chemical structure of the reacting proteins rather than by their biological origin.*

While gliadin and hordein differ in their ultimate composition, and also in the proportion of some of the products which they yield on hydrolysis, to such an extent that a difference in constitution is plainly shown, nevertheless they resemble one another so closely in physical properties and amino-acid make-up that *similarity* in chemical constitution is not at all improbable.

Glutenin, on the other hand, differs so distinctly from gliadin and hordein, both in solubility and amino-acid make-up, that there can be no question that it represents quite a different type of protein.

One striking feature of the reactions between gliadin and glutenin is that when the sensitizing dose is glutenin and the intoxicating dose also glutenin the reaction is less severe than when the sensitizing dose is gliadin and the intoxicating dose is glutenin. From this fact it is almost certain that the reaction between these proteins is not caused by contamination of the glutenin with traces of gliadin. Whether or not our preparations of gliadin were contaminated with traces of glutenin cannot be determined with absolute certainty, but the methods of preparation, and the differences in solubility of these two proteins make it far less probable that the gliadin contains any glutenin, than that the glutenin contains a trace of gliadin.

The most reasonable explanation of these results is that both gliadin and glutenin contain common groups which react with one another, and that the specificity of the anaphylaxis reaction is not dependent on the chemical make-up of the *entire protein molecule*. This view is further supported by the fact that *while animals sensitized with glutenin react to an intoxicating dose of gliadin, they*

<sup>1</sup> Cf. Osborne, *The Harvey Lectures*, 1910-1911, p. 67; also Abderhalden and Fodor, *Ztschr. f. physiol. Chem.*, 1912, 81, p. 1.

*do not react to an intoxicating dose of hordein.* These facts are in harmony with the evidence of two reacting elements in gliadin, which is also further shown by our saturation experiments with gliadin and hordein. In accord with these observations, the assumption does not appear unjustified that gliadin contains one group which reacts with hordein, and another which does not react with hordein, but does react with glutenin, this latter group being absent from hordein. In this connection the anaphylaxis reaction with a 14-member polypeptide, 1-leucyl-triglycyl-1-leucyl-octaglycyl-glycin, recently reported by Abderhalden<sup>1</sup> is of interest as suggesting the possibility of separate reactive groups within a single protein molecule.

Although our results do not give us an insight into the precise manner in which the anaphylaxis reaction is brought about, they do furnish the most definite indication yet obtained that the specificity of this reaction is determined by the chemical constitution of the proteins which cause it.

#### EXPERIMENTAL PART.

The proteins used in this investigation were gliadin and glutenin from wheat, gliadin from rye, and hordein from barley. Gliadin and glutenin together, and in approximately equal quantity, constitute about 80 per cent of the proteins of the seed of wheat (*Triticum vulgare*), from which they can be removed in the form of the familiar wheat gluten by washing the ground seed with water. Of this gluten, gliadin and glutenin form the chief part. Gliadin can be extracted from the gluten, or directly from the ground seed, by 70 per cent alcohol, in which it is freely soluble, but in which glutenin does not dissolve.

Our preparation of gliadin was extracted from gluten by 70 per cent alcohol and purified by repeatedly pouring its clear and concentrated alcoholic solution alternately into large volumes of cold water and absolute alcohol, until all the non-gliadin substances soluble in either of these solvents were, as far as possible, removed. The snow-white, friable product finally obtained was then digested with absolute ether, until everything soluble therein was extracted.

<sup>1</sup> *Ztschr. f. physiol. Chem.*, 1912, 81, p. 322.

The glutenin was brought into solution by means of very dilute alkali, precipitated by neutralizing its perfectly clear alkaline solution with hydrochloric acid, and the precipitate extracted with 70 per cent alcohol in order to remove gliadin. By five reprecipitations and digestions with 70 per cent alcohol, the gliadin was nearly all extracted, and then by repeated and prolonged digestions with 70 per cent alcohol until only the merest traces of anything was dissolved, the remainder was practically completely removed. The glutenin was then digested with absolute alcohol and ether, and obtained as a fine, snow-white powder.

The rye gliadin and the hordein were obtained by directly extracting the flour of rye (*Secale cereale*), and the flour of barley (*Hordeum vulgare*), respectively with 70 per cent alcohol, and purified by the same process as that employed in preparing the gliadin from wheat.

The similarities and differences between these three proteins are shown in the following table:<sup>1</sup>

	Gliadin Wheat	Gliadin Rye	Glutenin Wheat	Hordein Barley
<i>Solubility—</i>				
Water.....	Slightly sol.	Slightly sol.	Insol.	Slightly sol.
Abs. alcohol.....	Insol.	Insol.	Insol.	Insol.
Dil. alcohol.....	Very sol.	Very sol.	Insol.	Very sol.
Dil. aqueous acids.....	Sol.	Sol.	Sol.	Sol.
Dil. aqueous alkalis.....	Sol.	Sol.	Sol.	Sol.
<i>Ultimate composition—</i>				
Carbon.....	52.72	52.75	52.34	54.29
Hydrogen.....	6.86	6.84	6.83	6.80
Nitrogen.....	17.66	17.72	17.49	17.21
Sulphur.....	1.02	1.21	1.08	0.83
Oxygen.....	21.74	21.48	22.26	20.87
	100.00	100.00	100.00	100.00
<i>Products of hydrolysis—</i>				
Glycocoll.....	0.00	.....	0.89	0.00
Alanine.....	2.00	.....	4.05	1.34
Valine.....	3.34	.....	0.24	1.40
Leucine.....	0.62	.....	5.95	7.00
Proline.....	13.22	.....	4.23	13.73
Phenylalanine.....	2.25	2.70	1.97	5.48
Aspartic acid.....	0.58	.....	0.91	1.32
Glutamic acid.....	43.66	.....	23.42	43.20
Serine.....	0.13	.....	0.74	0.10
Tyrosine.....	1.20	1.19	4.25	1.67
Cystine.....	0.45	.....	0.02	?
Lysine.....	0.00*	0.00	1.02	0.00
Histidine.....	1.63	.....	1.76	1.28
Arginine.....	2.78	2.22	4.72	3.14
Tryptophane.....	1.00 (about)	Present	Present	Present
Ammonia.....	5.22	5.11	4.01	4.87
	84.08	.....	59.68	84.53

\* It is possible that even the purest gliadin may contain a very small amount of lysine. Cf. Osborne and Mendel, *Jour. Biol. Chem.*, 1912, 12, p. 473.

<sup>1</sup> For an account of the properties of these proteins and a discussion of the literature relating to them, see Osborne, *Ergebnisse der Physiologie*, 1910, 10, p. 62.

The results obtained when guinea-pigs were injected with hordein and gliadin dissolved in 0.1 per cent NaOH are shown in Table 1.

TABLE 1.  
INTERACTION OF HORDEIN AND GLIADIN.

Sensitizing Dose gm.	Second Dose 0.100 gm. (15-21 Days Later)	Result *	Third Dose 0.100 gm. (24-48 Hrs. Later)	Result
<i>Hordein, barley—</i>				
1. 0.010.....	Gliadin, wheat	Severe	Hordein, barley	Slight
2. 0.002.....	" "	"	" "	"
3. 0.001.....	" "	"	" "	"
4. 0.0002.....	" "	"	" "	Severe
5. 0.010.....	" "	"	" "	.....
6. 0.002.....	" "	Died, 25 min.	" "	.....
7. 0.001.....	" "	" 15 "	" "	.....
8. 0.0002.....	" "	Severe	Hordein, barley	Severe
9. 0.010.....	" "	"	" "	"
10. 0.002.....	" "	"	" "	.....
11. 0.001.....	" "	Died, 18 min.	" "	.....
12. 0.0002.....	" "	Severe	Hordein, barley	Severe
<i>Gliadin, wheat—</i>				
13. 0.010.....	Hordein, barley	Severe	Gliadin, wheat	Moderate
14. 0.002.....	" "	"	" "	Died, 2 min.
15. 0.001.....	" "	"	" "	Severe
16. 0.0002.....	" "	Doubtful	" "	Moderate
17. 0.010.....	" "	Severe	" "	"
18. 0.002.....	" "	"	" "	Severe
19. 0.001.....	" "	"	" "	Moderate
20. 0.010.....	" "	"	" "	Died, 2 hr.
21. 0.002.....	" "	Moderate	" "	Severe
22. 0.001.....	" "	Severe	" "	"
23. 0.0002.....	" "	Died, 35 min.	" "	.....
<i>Hordein, barley—</i>				
24. 0.010.....	Gliadin, rye	Severe	Hordein, barley	Severe
25. 0.002.....	" "	"	" "	Moderate
26. 0.001.....	" "	Died, 10 min.	" "	.....
27. 0.0002.....	" "	Severe	Hordein, barley	Moderate
<i>Gliadin, rye—</i>				
28. 0.010.....	Hordein, barley	"	Gliadin, rye	Severe
29. 0.002.....	" "	"	" "	"
30. 0.001.....	" "	"	Gliadin, rye	Moderate
31. 0.0002.....	" "	"	" "	.....

\* The terms used to describe the outcome of the experiments and also the methods employed in conducting them are explained in our previous paper, *loc. cit.*

Thus, of 12 guinea-pigs sensitized to hordein from barley (experiments 1-12), all reacted severely when injected after three weeks with gliadin from wheat, and of these, three died. The survivors were still sensitive to hordein from barley but they reacted much less strongly than they would if they had not been injected with gliadin. Of 11 guinea-pigs sensitized to gliadin from wheat, experiments 13-23, all but one reacted strongly to hordein from barley and one died; however, these reactions were distinctly less severe than the reactions usually obtained when the second injection is gliadin from wheat, for under these conditions a large proportion of the animals

die. After the guinea-pigs recovered they were still strongly reactive to gliadin from wheat—more so than were those treated with hordein-gliadin-hordein solutions to the second dose of hordein. With gliadin from rye and hordein from barley similar inter-reactions were also observed, as shown by experiments 24-31.

From the results of these experiments it is apparent that our preparations of gliadin from wheat or rye and hordein from barley are, from the standpoint of the anaphylaxis reaction, very closely related to one another. That gliadin and hordein are not identical proteins, however, has been shown by chemical comparisons. The results of these biological reactions are in harmony with the chemical reactions, since the symptoms caused by the heterologous proteins are definitely less severe than are those caused by the homologous proteins when used in corresponding doses. The fact that after recovery from an intoxicating dose of 0.100 gm. of the heterologous protein the animal is still more or less sensitive to the homologous protein, suggests the possibility that both gliadin and hordein contain reactive groups which are not saturated by the heterologous protein; for guinea-pigs after recovering from a 0.100 gm. dose of an homologous vegetable protein are usually entirely refractory to this same protein. The presence in both hordein and wheat gliadin of common and also specific antigens is thus indicated, so that the reaction after the first injection with the heterologous protein can be ascribed to the presence of a common antigen, and the reaction after the second injection of the original protein to another specific antigen not present in the heterologous protein.

If the inter-reactions between hordein and gliadin depend upon the presence in each protein of only homologous antigens—whether these be identical proteins common to each grain, or identical antigenic radicals in one and the same protein molecule—after sensitizing guinea-pigs with hordein, and then saturating them with gliadin, they should not then be sensitive to hordein, or vice versa. If, on the other hand, the two proteins also contain specific reactive groups not common to both of them, the animal, sensitized with one, and saturated with the other, should still react when injected with the protein with which it had originally been sensitized. Evidence of common and specific antigens in hordein and gliadin



ought, therefore, to be shown by sensitizing the animal to either of these proteins, and then saturating it with the other. Under such conditions the saturated animal should still react to the specific antigen belonging to the protein used in the intoxicating dose, and, as is shown in Table 3, this is the case.

In another article<sup>1</sup> one of us showed that the saturation principle, as applied to the precipitin and agglutinin reactions, can also be applied to the anaphylaxis reaction for the purpose of testing the presence of multiple antigens. Thus, if an animal is sensitized to two or more proteins, it can be made refractory to one of them by injection of a sufficient quantity of that protein in one or several non-fatal doses, but is still capable of reacting to the other protein or proteins with which it was sensitized. This use of the specific refractory condition following anaphylactic reactions is based on the view of Friedberger that this refractory condition depends upon exhaustion or saturation of the anaphylactic antibodies in the sensitive animal. Our extensive experience with this phase of anaphylaxis has been in entire harmony with this hypothesis, and the recent work of Weil and Coca<sup>2</sup> seems to be most convincing as to its correctness. A test was made of the applicability of this saturation principle for the detection of multiple antigens and antibodies in experiments with vegetable proteins, as our previous experience had been with animal proteins. The results are shown in Table 2.

Here the guinea-pigs were sensitized with a mixture of the globulin from squash-seed together with gliadin from wheat, and after three weeks they were saturated with one of these proteins, and found to be still reactive to the other, thus establishing the reliability of the saturation method for the detection of multiple antigens. One defect of this method, however, is that animals which have reacted to one antigen will not again react so strongly to another antigen as will animals which have not already been through a series of reactions. Animals sensitized either with globulin from the squash-seed, or with gliadin from wheat, will usually react very severely, often fatally, to an injection of 0.020 to 0.100 gm.

<sup>1</sup> Wells, *Jour. Infect. Dis.*, 1911, 9, p. 147.

<sup>2</sup> Weil and Coca, *Proc. Soc. Exper. Biol. and Med.*, 1912, 9, p. 147; *Ztschr f. Immunitätsf.*, 1913, 17, p. 141.

of the homologous protein, but, as is shown in Table 2, after recovering from reactions with either one of these proteins, our animals, which had been sensitized to both of these proteins, usually developed only moderate reactions with the other protein, severe reactions being rarely obtained. Although the reduced sharpness of the reaction somewhat lowers the value of this procedure for detecting mixtures of antigens, nevertheless we have applied it with definite results to the problem of the relation of hordein and gliadin, as shown in Table 3.

TABLE 2.

## SATURATION EXPERIMENTS WITH SQUASH-SEED GLOBULIN AND WHEAT GLIADIN.

Each animal had been sensitized with a mixture of 0.005 gm. squash-seed globulin and 0.005 gm. wheat gliadin three weeks before the first reinjection.

First Reinjection (21-Days After Sensitiza- tion) gm.	Result	Second Reinjection (24 Hrs. Later) gm.	Result	Third Reinjection (48 Hrs. Later) gm.	Result
<i>Squash-seed globulin—</i>					
1. 0.010 .....	Moderate	<i>Squash-seed globulin</i> 0.060	Moderate	0.100 gliadin	Severe
2. 0.020 .....	Severe	0.040	Slight	0.100 squash-seed globulin	o (control)
3. 0.020 .....	"	0.040	"	0.100 gliadin	Moderate
4. 0.020 .....	"	0.060	Moderate	0.100 "	Severe
5. 0.005 .....	"	0.040	"	0.050 squash-seed globulin	o (control)
6. 0.010 .....	Slight	0.050	"	0.060 gliadin	Moderate
7. 0.010 .....	Moderate	0.050	Doubtful	0.050 "	"
8. 0.010 .....	"	0.050	Moderate	0.050 "	"
<i>Gliadin—</i>					
9. 0.005 .....	Severe	<i>Gliadin</i> 0.050	Slight	0.100 "	o (control)
10. 0.010 .....	"	0.050	Moderate	0.100 squash-seed globulin	Moderate
11. 0.005 .....	Moderate	0.050	"	0.050 gliadin	Doubtful (control)
12. 0.010 .....	"	0.050	"	0.050 squash-seed globulin	Moderate
13. 0.010 .....	"	0.050	Slight	0.050 " " "	"
14. 0.010 .....	"	0.050	Doubtful	0.050 " " "	"

The results of the experiments given in Table 3 are so harmonious, that they are evidently reliable. Experiments 1-7 show conclusively that guinea-pigs sensitized with gliadin, and then saturated with gliadin by two subsequent injections of this protein, are no longer sensitive to hordein. Experiments 8-11 show equally distinctly that guinea-pigs sensitized with hordein and then saturated with hordein are rendered refractory to gliadin. The results given in Tables 1 and 3 show that gliadin from wheat and hordein from barley, although derived from plants belonging to different genera, react with one another as though they were one and the same

TABLE 3.

SATURATION EXPERIMENTS WITH GLIADIN FROM WHEAT OR RYE, AND HORDEIN FROM BARLEY.

Sensitizing Dose gm.	First Intoxicating Dose (15-21 Days Later) gm.	Result	Second Intoxicating Dose (24-48 Hrs. Later) gm.	Result	Third Intoxicating Dose (24-48 Hrs. Later) 0.100 gm.	Result
<i>Gliadin, wheat—</i>	<i>Gliadin, wheat—</i>		<i>Gliadin, wheat—</i>			
1. 0.010 .....	0.005	Slight	0.020	Slight	Gliadin, wheat	o (control)
2. 0.010 .....	0.010	Severe	0.050	o	Hordein, barley	o
3. 0.010 .....	0.005	Moderate	0.020	o	"	o
4. 0.010 .....	0.008	Severe	0.020	o	"	o
5. 0.010 .....	0.005	Slight	0.020	o	"	o
6. 0.010 .....	0.005	Moderate	0.020	o	"	o
7. 0.010 .....	0.007	Died	.....	.....	.....	.....
<i>Hordein, barley—</i>	<i>Hordein, barley—</i>		<i>Hordein, barley—</i>			
8. 0.010 .....	0.005	Moderate	0.050	o	Gliadin, wheat	o
9. 0.010 .....	0.010	Severe	0.100	o	"	o
10. 0.010 .....	0.010	"	0.100	Slight	"	o
11. 0.010 .....	0.005	Slight	0.100	o	"	o
	<i>Gliadin, wheat—</i>		<i>Gliadin, wheat—</i>			
12. 0.010 .....	0.020	Moderate	0.100	o	"	o (control)
13. 0.010 .....	0.040	"	0.100	o	Hordein, barley	Severe
14. 0.010 .....	0.020	"	0.100	o	"	"
15. 0.010 .....	0.020	"	0.100	o	"	"
	<i>Gliadin, rye—</i>		<i>Gliadin, rye—</i>			
16. 0.010 .....	0.020	Slight	0.100	Slight	"	Moderate
17. 0.010 .....	0.050	"	0.100	"	"	Severe
18. 0.010 .....	0.040	"	0.100	"	"	Moderate
19. 0.010 .....	0.050	"	0.100	"	"	Severe
	<i>Gliadin, wheat—</i>		<i>Gliadin, wheat—</i>			
20. 0.010 .....	0.020	Severe	0.100	o	Hordein, barley	Moderate
21. 0.010 .....	0.030	"	0.100	o	"	"
22. 0.010 .....	0.040	"	0.100	o	"	"
23. 0.010 .....	0.030	"	0.100	o	"	Died 90 min.
<i>Gliadin, wheat—</i>	<i>Hordein, barley—</i>		<i>Hordein, barley—</i>			
24. 0.010 .....	0.050	Moderate	0.100	Slight	Gliadin, wheat	Moderate
25. 0.010 .....	0.050	"	0.100	Doubtful	"	"
26. 0.010 .....	0.050	"	0.100	"	"	"
27. 0.010 .....	0.050	"	0.100	Slight	"	"
<i>Gliadin, rye—</i>						
28. 0.010 .....	0.100	Severe	0.100	Doubtful	Gliadin, rye	Severe
29. 0.010 .....	0.100	Moderate	0.100	o	Hordein, barley	o (control)
30. 0.010 .....	0.100	"	0.100	o	Gliadin, rye	Severe
31. 0.010 .....	0.100	"	0.100	o	"	"
<i>Hordein, barley—</i>	<i>Gliadin, rye—</i>		<i>Gliadin, rye—</i>			
32. 0.010 .....	0.100	Slight	0.100	o	Hordein, barley	"
33. 0.010 .....	0.100	Severe	0.100	o	Gliadin, rye	o (control)
34. 0.010 .....	0.100	"	0.100	o	Hordein, barley	Severe
35. 0.010 .....	0.100	"	0.100	o	"	"
<i>Gliadin, wheat—</i>	<i>Hordein, barley—</i>		<i>Hordein, barley—</i>			
36. 0.010 .....	0.050	"	0.100	Slight	Gliadin, wheat	"
37. 0.010 .....	0.050	"	0.100	Doubtful	Hordein, barley	o (control)
38. 0.010 .....	0.050	"	0.100	Slight	Gliadin, wheat	Moderate
39. 0.010 .....	0.060	"	0.100	"	"	Severe

protein; in other words, gliadin and hordein appear to contain one or more *common* antigenic groups.

Experiments I-11, however, do not show whether or not gliadin

and hordein also contain antigenic groups *specific* for each protein. Experiments 12-39 give strong evidence that specific groups are present in these proteins. Thus, when guinea-pigs were sensitized with one of these proteins, and then saturated with the other, it was found that the saturated animals were still sensitive to the protein with which they were originally sensitized. Since this result was obtained in every one of 24 experiments, any element of accident is excluded. As the animals sensitized with hordein and saturated with gliadin were still sensitive to hordein, the assumption is justified that hordein contains an antigen not present in gliadin, and conversely, gliadin contains an antigen not present in hordein. We must consequently conclude that gliadin (whether from wheat or rye) and hordein from barley contain both common and specific antigens. The reactions given in Table 3 may, therefore, be explained in the following way:

If we designate the common antigen as C, the specific antigen of gliadin as G, and the specific antigen of hordein as H, we may imagine the following situation: Gliadin will consist of GC and hordein of HC, and the reactions may be indicated by the following scheme:

First Injection	Second Injection	Result	Third Injection	Result	Explanation
(a) HC.....	GC	+	HC	+	1st + reaction is with antibody for C 2d + reaction is with antibody for H
(b) GC.....	HC	+	GC	+	
(c) HC.....	HC	++	GC	-	1st ++ reaction is with antibodies for H and C
					2d - reaction, because no antibodies are present for G or C
(d) GC.....	GC	++	HC	-	1st ++ reaction is with antibodies for G and C
					2d - reaction, because no antibodies are present for H or C

(1) An animal (a) sensitized to HC reacts to GC because of the presence of C in each protein, but the reaction is less severe than with HC, presumably because only one radical (C) is reacting.

(2) An animal (c) sensitized with HC and saturated with HC will not react with GC, because the antibodies for C are saturated. Similarly, if sensitized (d) with GC and saturated with GC it will

no longer react to HC, because of the exhaustion of the antibody for C.

(3) But if sensitized (*a*) with HC and saturated with GC it will still be sensitive to HC, since the antibodies for H remain, but the reaction will be less severe than if HC was injected for the first intoxicating dose since then both antigens react. Similarly (*b*) for the GC+HC+GC series.

Since the above conditions are exactly fulfilled in the experiments in this series, the conclusion seems warranted that *hordein and gliadin each contain distinct specific antigens as well as common antigens*. We must conclude from the results of our experiments, either that our preparations of gliadin and hordein each contain two different *proteins*, one of which is common to both preparations, or that they contain at least two reactive *groups*, one of which is common to both proteins, each of which groups behaves as a distinct antigen when injected into guinea-pigs. The chemical evidence supports the latter interpretation. We thus find that the group reactions which characterize species of close biological relationship, whether bacteria, plants, or animals, are also exhibited by purified proteins of similar chemical nature isolated from related species.

#### GLUTENIN EXPERIMENTS.

Experiments with glutenin gave the results shown in Table 4.

Animals sensitized with glutenin reacted severely to wheat gliadin and were then almost entirely refractory to glutenin. They did *not* react to hordein, and were *not* made refractory to glutenin by hordein. If sensitized with glutenin and then saturated with glutenin they were nearly, but not quite refractory to gliadin.

If sensitized with gliadin they reacted if anything, stronger to glutenin than if sensitized with glutenin, but were rendered little if at all refractory to gliadin by saturation with glutenin. When sensitized with hordein they did not react with glutenin, and were made only partly refractory to hordein.

Since glutenin and gliadin are the chief constituents of wheat gluten, and must be separated from one another to obtain the preparation used for this work, it might be assumed that the reactions here reported were caused by an incomplete separation of these

TABLE 4.  
REACTIONS WITH GLUTENIN FROM WHEAT.

Sensitizing Dose gm.	Second Dose (15-21 Days Later)	Result	Third Dose (24-48 Hrs. Later)	Result	Fourth Dose (24-48 Hrs. Later)	Result
<i>Glutenin, wheat:</i>						
1. 0.002...	Glutenin, wheat	Severe	Gladiin, wheat	o	.....	.....
2. 0.010...	" "	Moderate	Glutenin, wheat	o	Gladiin, wheat	Slight
3. 0.010...	" "	Slight	" "	o	" "	o
4. 0.010...	" "	o	" "	o	" "	o
5. 0.002...	" "	Moderate	.....	.....	.....	.....
6. 0.010...	" "	Slight	.....	.....	.....	.....
7. 0.010...	" "	Doubtful	Glutenin, wheat	o	Gladiin, wheat	o
8. 0.010...	" "	"	" "	o	" "	o
9. 0.002...	" "	Slight	" "	o	" "	o
10. 0.002...	" "	Moderate	" "	o	" "	o
11. 0.002...	Gladiin, wheat	Severe	Glutenin, wheat	Slight	.....	.....
12. 0.010...	" "	.....	Gladiin, wheat	o	.....	.....
13. 0.002...	" "	Died	.....	.....	.....	.....
14. 0.001...	" "	75 min.	.....	.....	.....	.....
15. 0.0002...	" "	Severe	Glutenin, wheat	Doubtful	.....	.....
16. 0.010...	" "	"	Gladiin, wheat	o	Glutenin, wheat	Doubtful
17. 0.010...	" "	"	" "	"	" "	"
18. 0.010...	" "	"	Glutenin, wheat	Slight	.....	.....
19. 0.010...	" "	o	" "	Moderate	.....	.....
20. 0.010...	Gladiin, rye	o	" "	Doubtful	.....	.....
21. 0.002...	" "	o	" "	Slight	.....	.....
22. 0.001...	" "	Moderate	" "	Moderate	.....	.....
23. 0.0002...	" "	Slight	" "	o	.....	.....
24. 0.001...	" "	o	" "	o	.....	.....
25. 0.001...	" "	"	" "	o	.....	.....
26. 0.010...	" "	Slight	Gladiin, rye	o	Glutenin, wheat	Slight
27. 0.002...	" "	"	" "	o	" "	"
28. 0.001...	" "	"	" "	o	" "	"
29. 0.0002...	" "	Died	.....	.....	.....	.....
		30 min.				
<i>Gladiin, wheat:</i>						
30. 0.010...	Glutenin, wheat	Severe	Gladiin, wheat	Moderate	.....	Slight
31. 0.002...	" "	Moderate	.....	.....	.....	.....
32. 0.0002...	" "	Severe	.....	.....	.....	.....
33. 0.010...	" "	.....	Gladiin, wheat	Moderate	.....	.....
34. 0.002...	" "	Died	.....	.....	.....	.....
		2 hrs.				
35. 0.001...	" "	Severe	Glutenin, wheat	o	.....	.....
36. 0.0002...	" "	"	Gladiin, wheat	Moderate	.....	.....
37. 0.010...	" "	Moderate	Glutenin, wheat	o	Gladiin, wheat	Slight
38. 0.010...	" "	"	" "	o	" "	"
39. 0.010...	" "	Severe	" "	o	" "	Moderate
40. 0.020...	" "	"	" "	o	" "	Severe
41. 0.010...	" "	Moderate	" "	o	" "	Moderate
42. 0.020...	" "	"	" "	o	" "	Severe
43. 0.010...	" "	Severe	" "	o	" "	Moderate
44. 0.002...	" "	"	" "	o	" "	"
45. 0.002...	" "	Moderate	" "	o	" "	"
<i>Gladiin, rye:</i>						
46. 0.010...	" "	"	" "	Slight	Gladiin, rye	Severe
47. 0.002...	" "	Slight	" "	Doubtful	.....	.....
48. 0.001...	" "	"	" "	o	Gladiin, rye	Severe
49. 0.0002...	" "	Moderate	" "	o	.....	.....
50. 0.010...	" "	"	" "	o	Gladiin, rye	Slight
51. 0.002...	" "	Severe	" "	o	" "	"
52. 0.001...	" "	"	" "	o	" "	"
53. 0.0002...	" "	Moderate	" "	o	" "	"
<i>Hordein, barley:</i>						
54. 0.010...	" "	o	Hordein, barley	Slight	.....	.....
55. 0.010...	" "	"	" "	Moderate	.....	.....
56. 0.010...	" "	o	" "	"	.....	.....
57. 0.010...	" "	o	" "	Severe	.....	.....
58. 0.010...	" "	Doubtful	" "	o	Hordein, barley	Slight
59. 0.002...	" "	"	" "	o	" "	"
60. 0.001...	" "	o	" "	o	" "	"
61. 0.0002...	" "	o	" "	o	" "	"

TABLE 4—Continued.

Sensitizing Dose gm.	Second Dose (15-21 Days Later)	Result	Third Dose (24-48 Hrs. Later)	Result	Fourth Dose (24-48 Hrs. Later)	Result
<i>Glutenin, wheat:</i>						
62. 0.010...	Hordein, barley	o	Glutenin, wheat	Slight	.....	.....
63. 0.002...	" "	o	" "	"	.....	.....
64. 0.001...	" "	o	" "	"	.....	.....
65. 0.0002...	" "	o	" "	"	.....	.....
66. 0.010...	" "	o	" "	"	.....	.....
67. 0.010...	" "	o	" "	"	.....	.....
68. 0.010...	" "	o	" "	"	.....	.....
69. 0.010...	" "	o	" "	"	.....	.....
70. 0.005...	" "	o	Hordein, barley	o	Glutenin, wheat	Slight
71. 0.001...	" "	o	" "	o	" "	Doubtful
<i>Mixture of glutenin and gliadin, wheat:</i>						
72. 0.010...	" "	Moderate	Glutenin, wheat	Slight	Gliadin, wheat	Severe
73. 0.002...	" "	"	" "	"	" "	Moderate
74. 0.001...	" "	Severe	" "	Moderate	" "	"
75. 0.0002...	" "	"	" "	"	" "	Severe

## SUMMARY.

Glutenin vs. glutenin: 11 experiments, severe 1, moderate 3, slight 3, doubtful 1, no symptoms 3.

Glutenin vs. wheat gliadin: 8 experiments, severe 7, fatal 1.

Glutenin vs. rye gliadin: 10 experiments, moderate 1, slight 4, fatal 1, no symptoms 4.

Glutenin vs. hordein: 10 experiments, no symptoms in any.

Wheat gliadin vs. glutenin: 10 experiments, fatal 1, severe 9, moderate 6.

Rye gliadin vs. glutenin: 8 experiments, severe 2, moderate 4, slight 2.

Hordein vs. glutenin: 8 experiments, doubtful 1, none 7, no symptoms 5.

Sensitized with glutenin and saturated with glutenin: protects vs. wheat gliadin.

Sensitized with glutenin and saturated with hordein: not protected vs. glutenin.

Sensitized with gliadin and saturated with glutenin: only slight protection.

Sensitized with glutenin + wheat gliadin react well with hordein, also with glutenin and wheat gliadin.

proteins. We have already given our reasons for believing that the separation was nearly complete, and that no more than traces of gliadin were present in our preparation of glutenin, or of glutenin in the preparation of gliadin. If this assumption is correct, our experiments justify the conclusion that gliadin and glutenin contain common reacting groups, for those animals sensitized by the smallest quantity of either one of these proteins reacted quite as severely, when intoxicated with the heterologous protein, as did those intoxicated with the homologous protein. In other words, gliadin and glutenin react with one another almost as if they were identical proteins, although all of the chemical evidence, especially that relating to their amino-acid make-up, shows them to be distinctly different proteins. The fact that hordein, which readily reacts with gliadin, fails to react with glutenin also supports this view, for, if the glutenin preparations contained sufficient gliadin

to render the animals sensitive to gliadin we should certainly expect them to show some symptoms at least when subsequently treated with hordein.

These experiments give no clue as to whether or not the gliadin preparation is contaminated with glutenin, and at present there appears to be no means whereby this question can be settled beyond a doubt. The commonly accepted opinion that gliadin yields no lysine on hydrolysis, whereas glutenin does, might be offered as evidence on this point. One of us, however, has recently found<sup>1</sup> that a supposedly pure preparation of gliadin, which, when tested according to Kossel's method, gave no indication of yielding any lysine, did, in fact, yield a very small amount, which could be detected when changes in Kossel's method were made with a view to isolating very small quantities of the picrate of this amino-acid. Investigations now in progress have given, as yet, no evidence indicating the presence of glutenin in the preparation of gliadin under examination.

#### LOCAL PERITONEAL REACTIONS.

In the course of our experiments, involving repeated injections of foreign proteins into the same animal, we have noticed a somewhat inconstant, but often striking phenomenon, to which we wish to call attention. When an animal which has been given the large second or intoxicating dose of the vegetable protein into the peritoneum, is given 24 to 72 hours later another intraperitoneal injection of the same protein, to which it is now, as a rule, entirely refractory as regards anaphylactic reaction, it may exhibit symptoms of a severe, but very transient peritoneal irritation, although the previous dose of the same protein had had no similar effect. About 15 to 60 seconds after injection the animal dashes about madly for a few seconds, jumps up and down, arches the back as if trying to relieve intraperitoneal pressure, and seems in great distress for one-half to two minutes; the symptoms cease quickly, and after one or two minutes more the animal seems entirely well. Apparently the reaction is, at least partly, specific, for unless the two injections are with the same protein the effect is not observed.

<sup>1</sup>Osborne and Mendel, *Jour. Biol. Chem.*, 1912, 12, p. 473.



Since first observing this phenomenon we have made note of its occurrence, and find that it is usually, but not always, exhibited, and in one set of experiments it may be marked and yet be entirely missing in a duplicate set made at another time, and that there are many other variations and discrepancies, so that we cannot as yet interpret it. Possibly it is in the nature of a local sensitization and reaction.

#### SUMMARY.

1. The preparations of proteins used for the experiments described were very carefully purified in order to separate them as completely as possible from all other proteins.

2. These proteins were hordein from barley, glutenin from wheat, and gliadin from both wheat and rye. Chemical investigations have established such marked differences between hordein, glutenin, and gliadin that they are commonly regarded as well-established individual proteins. Between gliadin from wheat and gliadin from rye, no difference has been observed sufficient to justify the assumption that these are different proteins.

3. Guinea-pigs sensitized with gliadin from wheat, or rye, give strong anaphylactic reactions with hordein from barley, but these are not as strong as the reactions obtained with the homologous protein. Similar results are obtained if the sensitizing protein is hordein and the second injection is gliadin. We here have a common anaphylaxis reaction developed by two chemically distinct, but similar, proteins of different biological origin, thus indicating that the specificity of this reaction is determined by the chemical constitution of the protein rather than by its biological origin. This is in harmony with the fact that chemically closely related proteins have, as yet, been found only in tissues that are biologically nearly related.

4. Complete protection to subsequent injection of the homologous protein was not afforded by a reaction to the heterologous protein, thus indicating the presence of two or more individual proteins in the preparations of gliadin and hordein, one of which is common to both, or else the presence in gliadin and hordein of both common and specific reactive groups. The chemical evidence is in favor of the latter conclusion.

5. The foregoing indications are supported by saturation experiments, which show that when guinea-pigs are sensitized with either gliadin or hordein, and then saturated with the heterologous protein, they still react strongly when injected with the homologous protein.

6. Gliadin and glutenin react anaphylactically with one another, although chemical comparisons have shown them to be proteins of distinctly different types. Evidence was obtained that the reactions between these proteins should not be ascribed to contamination of the preparations with one another, i.e., to an incomplete separation of the two. The conclusion appears justified that these chemically distinct proteins contain common reactive groups.

7. Guinea-pigs sensitized with glutenin do not react anaphylactically with hordein, thus showing that the reaction between gliadin and glutenin is not caused by an incomplete separation of these latter proteins, but by reactive groups common to gliadin and glutenin, but absent from hordein.

8. From the results of these experiments it seems probable that the entire protein molecule is not involved in the specific character of the anaphylaxis reaction, but this is developed by certain groups contained therein, and that one and the same protein molecule may contain two or more such groups. It may well be that the intact protein molecule is involved in the reaction (for there is but little evidence that anything less than an intact protein molecule is capable of producing the typical reaction), but that certain groups determine the specificity. Such a conclusion cannot be accepted as final until we have some means whereby the chemical individuality of a protein can be established. Until then the possibility will remain that our so-called pure preparations of proteins consist of mixtures, or combinations, of proteins which have thus far resisted all efforts to separate them. In this latter case the reactions here attributed to groups in one protein molecule might be caused by individual proteins contained in the preparations made by the methods now in use.

9. These experiments demonstrate that the "group reactions," characteristic of biological reactions between closely related species,

which usually have been interpreted as indicating the presence in related organisms of identical as well as distinct proteins, can really be exhibited by single isolated proteins from related organisms. In other words, biological relationship and chemical relationship seem to be much the same.

10. Attention is also called to certain other observations; (*a*) that animals sensitized with two proteins will, as is well known, react with either, and that after recovery from reaction with one protein the reaction given with the second protein is less severe than it would be if the animal had not already passed through an anaphylactic intoxication; (*b*) that after injection with an intoxicating dose of a vegetable protein, another injection with the same protein 24 to 72 hours later, when the animal is usually insusceptible, so far as constitutional symptoms are concerned, often produces a severe, transient peritoneal irritation, which seems to be in the nature of a specific local reaction.

## A NOTE ON THE MAINTENANCE OF VIRULENCE BY BACILLUS ABORTUS, BANG.\*

FRANK M. SURFACE.

(From the Biological Laboratory of the Kentucky Agricultural Experiment Station, Lexington, Kentucky.)

In the fall of 1911 the writer brought to this country three cultures of *Bacillus abortus*, Bang, which had been kindly given him by Professors Jensen and Holth of the Veterinary Serum Laboratory in Copenhagen. These cultures were marked as being in the seventh to the ninth generation. I have no notes as to how long these strains had been grown in the laboratory before I received them. However, from various conversations with Dr. Holth I have reason to believe that they had been in the laboratory for more than a year.

As is well known, the Bang bacillus, when first isolated, does not grow readily in the atmospheric pressure of the air. After it has been grown for some generations on culture media it may be induced to grow in the air. These Danish cultures had become accustomed to growing in the air for some time before I received them. Since that time they have never been grown in any other way.

During 1912 these cultures, with others, were repeatedly transferred from agar to agar or from agar to plain peptone broth and then back to agar. It has been found that this latter procedure often revives a somewhat sluggishly growing culture so that it grows much more luxuriantly than before. Thus these Danish cultures had been grown in the laboratory for at least two years and probably longer. During this time they were not passed through animals or in any way handled so as to increase their vigor except the occasional alteration from agar to broth as noted above. It was thought that they had probably lost their virulence, so far as causing abortion in cattle was concerned.

In connection with some experiments on the rate of production of antibodies after inoculation with killed cultures of the abortion bacilli, I inoculated one cow with a mixed culture of these Danish

\* Received for publication March 12, 1913.

strains. These organisms were washed from agar slants with salt solution and one-half of one per cent of carbolic acid added. The mixture was not subjected to heat, as is the usual method, but cultural tests made at that time failed to show any growth. From this it was concluded that the carbolic acid had been sufficient to kill the bacteria.

Since that time I have been able to demonstrate that one-half of one per cent carbolic acid is not sufficient to kill the abortion bacilli. Such treatment appears to retard their growth slightly, but otherwise they are as vigorous as before. I failed, for some reason, to obtain a growth in the test mentioned above, although, as subsequent results showed, the organisms were probably not dead.

The cow used in this experiment was a grade Jersey purchased in July, 1912, for experimental purposes. According to her previous owner she had never aborted and came from a farm where there had been no abortion. She was bred June 10, 1912, i.e., before coming to the Experiment Station.

During the summer and autumn her blood was tested at frequent intervals for abortion antibodies by both the agglutination and complement fixation tests.<sup>1</sup> At no time could abortion antibodies be demonstrated in her blood except as noted in the table below.

On November 25, 1912, she received subcutaneously, 20 c.c. of a dilute suspension of the carbolized but unheated Danish cultures referred to above. This suspension showed a count of approximately 500,000 bacteria to the cubic centimeter.<sup>2</sup> Thus there were injected in all, about 10,000,000 bacteria, supposedly dead. The history of this cow from just before the injection to the time of writing is given below in tabular form.

Nov. 14, 1912—Blood test gave no reaction.

Nov. 25, 1912—Subcutaneous injection of abortion bacilli as noted above. Blood test gave no reaction.

Dec. 2, 1912—Blood test gave no reaction.

Dec. 9, 1912—Blood test gave slight reaction. Fixation of complement with 0.05 c.c. serum but not with less amounts.

<sup>1</sup> The technic used in these tests has been fully described in a previous paper—*Ky. Agric. Exper. Sta., Ann. Rpt.*, 1912, Bull. 166, p. 303.

<sup>2</sup> This count was made after the method described by Callison, *Jour. Med. Research.*, 1912, 27, p. 225.

- Dec. 20, 1912—Blood test showed strong reaction—agglutination with 0.001 c.c. of serum. Fixation of complement with 0.005 c.c. serum.
- Jan. 10, 1913—Showed signs of aborting.
- Jan. 13, 1913—Blood test gave strong reaction. Same titre as December 20, 1912.
- Jan. 16, 1913—Aborted a well-developed calf. Large amount of typical yellowish exudate around the cotyledons. Abortion bacilli isolated. (Cf. below.)
- Feb. 10, 1913—Blood test gave strong reaction.

The cow was kept during this period in a barn with other cows where abortion had been very prevalent. Under such a condition she might very easily have become infected from such sources. The following observations, however, do not support this view.

A few hours after the abortion, the afterbirth was removed from the cow by a veterinarian. In this way I was able to obtain a number of cotyledons, showing typical exudate, which had had little chance to become contaminated with other organisms. Material from the foetal stomach and intestines was also obtained free from contamination. Plain agar plates were inoculated from this material. After 24 hours at 37° C. they gave no growth except a very few contaminating colonies from the cotyledons. About half the plates were then placed in a Novy jar with *Bacillus subtilis* after the method of Nowac.<sup>1</sup> On the second day it was noted that the plates remaining in the air developed a fine, opalescent, dew drop growth characteristic of the Bang bacillus. This growth increased within the next day or two so that a very rich growth, and in the majority of plates, a pure culture appeared. By microscopical examination and also by using it as antigen in a complement fixation test with an immune serum, this growth proved to be the *B. abortus*. After four days the plates were removed from the Novy jar and these also had a rich growth of the same organism. However, the growth obtained in the Novy jar under reduced pressure of oxygen was in no respect better than that obtained in the free air. Those who have had experience in isolating this organism from aborted material will realize that it is certainly very unusual to find it growing in the free air in the first generation, at least on the second day of incubation. Fabyan<sup>2</sup> however, states

<sup>1</sup> *Ann. de l'Inst. Pasteur*, 1908, 22, p. 541; Good, E. S., *Ky. Agric. Exper. Sta.*, 1912, *Bull.* 165, p. 227

<sup>2</sup> *Jour. Med. Research*, 1912, 26, p. 441.

that sometimes this organism will grow in the air when first isolated. He says: "It is probable that strains will be found to vary more or less in their relation to oxygen and to the kinds of culture media usually employed. . . . For instance, culture II, *a* and *b*, we were able to isolate without *B. subtilis* directly from guinea-pigs inoculated with the original material. In this instance, the tissues of two guinea-pigs were broken up as described, and spread over slants of ordinary agar containing one-half to one cubic centimeter of defibrinated guinea-pigs' blood. After a period of some four days, pure cultures were obtained."

In my own experience which has covered the examination of a considerable number of aborted foeti I have never been able to obtain a growth of this organism on the surface of agar from material direct from an aborting cow or foetus. It will be noted that Fabyan obtained this surface growth after passage through a guinea-pig. Whether this modified the organism in any way I am not able to say. It will further be noted that in the case I have described above I obtained a growth on plain agar (without any serum) and on the second day of incubation. These characteristics correspond very well with those of the Danish strains which were injected. Fabyan<sup>1</sup> also makes the following statement regarding this organism: "It is an interesting fact that after the organism has adapted itself to ordinary aërobic conditions it does not lose this characteristic by further passage through guinea-pigs. Some of our guinea-pigs so inoculated have lived fifteen to twenty weeks and yet upon making cultures from the spleen *B. abortus* developed even at room temperature."

I may further say that the strain of *B. abortus* which is prevalent in this portion of the Experiment Station herd, is very difficult to grow under any condition. I have frequently been unable to grow it at all, although from microscopical examination I was certain that the organism was present in the material. It is also very difficult to accustom this strain to grow in the free air.

From these considerations I think we may reasonably infer that the abortion in this case was due to the injection of the carbolized but unheated Danish culture. Thus these organisms which have

<sup>1</sup> *Loc. cit.*

been grown in the laboratory for two years and probably longer, have not lost their virulence so far as causing abortion in cattle is concerned.

We may also note that after the subcutaneous injection we were able to detect abortion antibodies after 14 days but not at the end of 7 days. At the end of 25 days the blood showed its strongest reaction. It has maintained this titre up to the time of writing. The abortion occurred 52 days after the inoculation. These observations agree reasonably well with the data obtained by MacFadyen and Stockman<sup>1</sup> and others with experimentally inoculated cows.

#### SUMMARY.

The subcutaneous injection of a cow with 20 cubic centimeters of a dilute mixture of three Danish strains of *B. abortus* which had been grown in the laboratory for more than two years, produced a typical abortion 52 days after the injection. The mixture had not been heated but contained one-half of one per cent of carbolic acid. The cultural test made at the time revealed no growth from this mixture. Since that time it has been found that one-half of one per cent of carbolic acid is not sufficient to kill this organism.

Cultures of this organism were isolated from the afterbirth and foetus. These cultures grew readily in the air. Growth appeared on plain agar on the second day of incubation. In these respects they agreed with the Danish strains injected and were totally different from the strain of this organism prevalent in the Experiment Station herd.

Blood tests at various intervals showed the appearance of antibodies 14 days after inoculation or about five weeks before the abortion.

Thus after more than two years' growth under laboratory conditions this organism has retained its original virulence.

<sup>1</sup> *Rpt. of Abortion Committee*, Bd. of Agric. and Fisheries of Great Britain, London, 1909, pt. 1.



## EXPERIMENTAL STREPTOCOCCAL ARTHRITIS IN RABBITS.\*

A SECOND STUDY DEALING WITH STREPTOCOCCI FROM THE MILK EPIDEMIC OF SORE THROAT IN CHICAGO, 1911-12.†

LEILA JACKSON.

(From the Laboratory of St. Luke's Hospital, Chicago, Illinois.)

In the article by Fritz Meyer<sup>1</sup> the literature of experimental arthritis is so thoroughly reviewed up to 1901 that only brief mention of the early investigations need be made. They chiefly concern clinical and bacteriological studies. The descriptions of the gross alterations are rather incomplete. Some exception to this is found in the accounts by Bannatyne, Wohlmann and Blaxall<sup>2</sup> of the changes produced in the joint by intravenous inoculation of an organism isolated from the inflammatory exudate in the joints of patients with rheumatism, an organism which they regarded as a bacillus. The description of the changes produced in rabbits' joints is as follows:

"In the joints we found proof that the microorganisms grow and propagate, doing so not only in the synovial membrane, ligaments, etc., but also in the bone marrow and cartilaginous structures. Their presence gives rise to acute inflammatory changes leading to ulceration, erosion and destruction of the hard as well as the soft joint tissues. Our specimens are so few that this is only a provisional statement."

Meyer<sup>3</sup> produced an arthritis in rabbits with organisms isolated from rheumatic angina in 12 patients. The pathological anatomy is not described. Beaton and Ainley Walker<sup>4</sup> obtained joint affections in rabbits by the injection of micrococci isolated from cases of rheumatism, and of chorea and endocarditis in rheumatic subjects. Concerning this micrococcus they say: "culturally this organism resembles a streptococcus." Shaw<sup>5</sup> using organisms from three different sources, *Streptococcus aus chorea* obtained

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† See "Experimental Rheumatic Myocarditis," *Jour. Infect. Dis.*, 1912, 11, p. 243.

<sup>1</sup> *Verhandl. d. cong. f. inn. Med.*, 1901, 19, p. 452.

<sup>2</sup> *Lancet*, 1896, 1, p. 1120.

<sup>3</sup> *Op. cit.*

<sup>4</sup> *Brit. Med. Jour.*, 1903, 1, p. 237.

<sup>5</sup> *Jour. Path. and Bacteriol.*, 1903, 9, p. 159.

from Wassermann, Ainley Walker's *Micrococcus rheumaticus* and Poynton's *Diplococcus rheumaticus* produced practically all the lesions clinically associated with an attack of rheumatism. Similarly Beattie<sup>1</sup> speaks of intravenous inoculation of *Diplococcus rheumaticus* recovered from congested areas about the knee joint in a case of rheumatic fever. Cole<sup>2</sup> described the changes produced in rabbits by the injection of seven different strains of streptococci from sources other than acute rheumatism without, however, attempting to follow the changes as they develop from early to late. Harris<sup>3</sup> produced arthritis in rabbits following the injection into the circulation of streptococci isolated from human sepsis and pharyngitis. Koch<sup>4</sup> produced a condition resembling articular rheumatism in young dogs by intravenous injection of *Streptococcus longus*. He emphasizes the predilection epiphyseal cartilages and the regions about them possess for initial changes.

In a number of articles by Poynton and Paine there are references to the changes in the joints. In an early publication<sup>5</sup> they state:

"There was also exudation into the tendon sheaths around the affected joints and the connective tissue near the larger joints had the gelatinous appearance of the nodule in man. We demonstrated the diplococci in the valves, pericardium, joint exudate, liver, kidneys, connective tissues around the joints and in large numbers in the lungs and pleurae." In the same article, referring to the bacteria in the tissues about the blood vessels in the pericardium and myocardium, they say: "In the earliest stages these organisms can be discovered in these tissues without having apparently caused any reaction, but a proliferation of connective tissue cells and a free exudation of leukocytes is usually easily recognized as a result of their presence." In another publication<sup>6</sup> there is the following description of the changes in the joint of a rabbit killed 10 weeks after inoculation. "The right knee was enlarged and the articular surfaces distinctly though slightly flattened. The edges of the articular surfaces were rounded, and if compared with the corresponding joint, thickened, giving rise to some lipping. A thickening on the inner condyle of the femur was especially noteworthy. In two places on the outer condyle of the femur the bone was eroded and the cartilage destroyed. There was both bone formation and bone destruction. The cartilage on the tibia had lost its natural gloss, and with a lens was seen to be roughened and pitted. . . . The atrophy of the muscles around the joint was very evident. The joint

<sup>1</sup> *Jour. Path. and Bacteriol.* 1904, 9, p. 272.

<sup>2</sup> *Jour. Infec. Dis.*, 1904, 1, p. 714.

<sup>3</sup> *Trans. Chicago Path. Soc.*, 1905, 4, p. 303.

<sup>4</sup> *Ztschr. f. Hyg. u. Infektionskrankh.*, 1912, 72, p. 321.

<sup>5</sup> *Lancet*, 1900, 2, p. 861.

<sup>6</sup> *Trans. Path. Soc.*, London, 1902, p. 221.

capsule was not greatly thickened, but the ligaments, especially the crucial, were opaque and swollen. . . . Whatever the causes of rheumatoid arthritis may be this appears to be quite certain, that if it is the result of an infection, the paths of access are far distant from the joints, and these structures are damaged because they are particularly susceptible, and not because there is direct invasion by microorganisms as in traumatic arthritis, the result of a wound." In a later article<sup>1</sup> they say: "The organism is deposited in the areolar tissue below the free margin of the synovial membrane," and that it "multiplies and flourishes in the local lesion and the blood stream is only a channel of conduction." Considering the origin of the inflammatory process in the joints, Poynton<sup>2</sup> remarks: "There is as can be imagined considerable difficulty in discovering with exactitude in which structure the process commences, but as far as the infective forms are concerned the balance of evidence points to the synovial membrane as the starting point in the majority of instances. The vascular supply is very considerable and the synovial membrane in a condition of acute inflammation." Of the infective agent, he says: "It is carried into the terminal capillaries by the blood stream and escaping finds itself in the retiform tissue of the membrane." He further observes: "The condition of the blood vessels in the capsule and synovial membrane is also of interest. In these protracted cases there is a perivascular exudation and later a perivascular fibrosis which interferes with the circulation in these structures and thus impairs the recuperative power."

There is still more of detail in the recent article by Coombs, Miller and Kettle.<sup>3</sup> The alterations in the joints of a rabbit, killed six days after intravenous inoculation with streptococci isolated from the vegetations on the tricuspid valve in a case of rheumatism, are thus described:

"The synovial membrane shows the formation of many fibroblasts, also new capillaries. The vascular endothelia are proliferating in some instances so much as to obliterate the vessel. In places there are groups of cells with much cytoplasm and one or more clear, transparent nuclei. Several vessels are completely occluded by thrombosis, others by masses of homogeneous material mixed with organisms. The whole of the synovial membrane and periarticular tissue are infiltrated with eosinophil leukocytes, which are also poured out freely into the cavity of the joint. A few of these are degenerating, but for the most part they have kept their structure well."

Concerning changes similar but somewhat more advanced in the joints of a rabbit which received two intravenous injections, the first producing arthritis from which the animal recovered, the second given four months and nine days later resulting in arthritis and death 16 days after the last inoculation, they say:

<sup>1</sup> *Lancet*, 1905, 2, p. 1760.

<sup>2</sup> *Med. Press and Circular*, 1907, p. 360.

<sup>3</sup> *Lancet*, 1912, 2, p. 1209. These authors duplicate my observations on the experimental production of myocardial lesions in rabbits, similar to those found in human "rheumatic myocarditis," by intravenous injection of streptococci.

"Throughout the synovial membrane there is a diffuse fibroblastic reaction, very intense but without leukocytosis. Practically all the small vessels and most of the large ones display a definite endothelial proliferation. There are also fibroblastic rings about them. The endovascular reaction has in one or two places ended in thrombosis; but as a rule it reaches its highest expression in the formation of typical 'submiliary nodules.'" With regard to the condition in another joint they state: "In the joint the similarities and differences between clinical and experimental rheumatism are exemplified. The reaction in the synovial tissues was mainly gathered together into rounded masses curiously like the subcutaneous nodes seen in rheumatic children. Some of these consist entirely of (a) many blood vessels most of them with swollen and proliferating endothelium, sometimes so far developed as to constitute a 'submiliary nodule' in embryo; (b) fibroblasts lying in every plane, but especially arranged in whorls around blood vessels; (c) a diffuse but moderate leukocytosis. In others (and here it is that the characteristics of the experimental type of lesion are exemplified), there are large masses which seem to consist of streptococci lying in the center of the inflamed areas; round these masses the leukocytosis was more intense and there were definitely necrotic changes."

Joint affections were frequent in the milkborne epidemic<sup>1</sup> of sore throat in Chicago during 1911 and 1912. Reference to the frequency of arthritis as a sequence of such streptococcus infections of the throat is made by a number of writers<sup>2</sup> not only concerning the Chicago epidemic but also in others. The occurrence of inflammation in the joints of rabbits inoculated with organisms isolated by Dr. Davis in the course of the Chicago epidemic has been mentioned in previous articles.<sup>3</sup> The material accruing from the experiments was nearly all fixed in Zenker's fluid and after decalcification the sections were stained by various standard methods, hematoxylin and eosin, phosphotungstic acid hematoxylin, polychrome methylene blue and eosin, the Giemsa and Wright methods for bacteria, etc.

*Changes in two rabbits killed two and four hours respectively after single injections of the 24-hour growth at 37° C. on two blood agar slants of a streptococcus (236) from the sero-fibrinous peritoneal exudate found at necropsy, and producing arthritis in rabbits. The peritonitis was a sequence of sore throat during the milk epidemic.*—In sections of the joints of these two rabbits, streptococci are found in some of the capillaries of the synovial membrane. The organisms are not in sufficient numbers to occlude the vessels but are scattered rather evenly among the blood cells. Aside from this there are no changes in the tissues.

<sup>1</sup> J. A. Capps and J. L. Miller, *Jour. Am. Med. Assn.*, 1912, 58, p. 1912.

<sup>2</sup> Robinson, *Practitioner*, 1884, 32, p. 467; Hall, *Glasgow Med. Jour.*, 1890, 34, p. 241; Sömme, *Lancet*, 1908, 1, p. 1707; Winslow, *Jour. Infect. Dis.*, 1912, 10, p. 73; Davis, *Illinois Med. Jour.*, 1912, 22, p. 585; Hamburger, *Bull. Johns Hopkins Hosp.*, 1913, 24, p. 1, (one case).

<sup>3</sup> *Jour. Am. Med. Assn.*, 1912, 58, p. 1283; *Jour. Infect. Dis.*, 1912, 11, p. 243.

*Changes in a rabbit killed 10 hours after a single intravenous injection of the same streptococcus (236), using the growth from four blood agar slants after 24 hours at 37° C.*—Microscopic preparations of the carpal and tarsal joints were examined. In places the capillaries of the synovial membrane contain streptococci which are also present in small numbers in the joint cavity. In the latter location they occur in pairs or short chains of four or six organisms surrounded by a small amount of a pink-staining material without cells. This material does not give the staining reaction of fibrin. In sections of the carpal joint a few vessels in the diaphysis of the radius near the epiphyseal cartilage are crowded with polymorphonuclear leukocytes and in the same region and just at the edge of the cartilage is a small vessel filled with cocci. There is no necrosis of the tissues. Also in sections of the carpal joint, many of the vessels in a triangular portion of the tibia with one side at the outer anterior surface of the bone and another along the epiphyseal cartilage, contain bacteria which completely occlude the vessels beneath the periosteum.

*Changes in a rabbit killed 24 hours after a single injection of the growth on two blood agar slants of the same streptococcus (236).*—Sections from both carpal joints were examined. The cavities of one joint are not involved; those of the other contain a moderate amount of exudate (Fig. 1) in which there are many organisms. The cells present in this exudate are chiefly polymorphonuclear leukocytes. There are a few places in the synovial membrane where the inner layer of cells is necrotic but in general this layer of cells appears fairly well preserved. The blood vessels contain a moderately increased number of polymorphonuclear leukocytes. In a few places small vessels are found containing bacteria and about them there is an infiltration of polymorphonuclear leukocytes and mononuclear cells. There is necrosis of the fibro-cartilaginous zone in some places. The changes being so alike in both joints, one description will answer for both. There is little or no involvement of the carpal bones, but the epiphyseal cartilage, the cancellous bone and the bone marrow on either side of the cartilage and the periosteum in this region of the long bones, are the seat of many lesions. There is a triangular region in the diaphysis of one of these bones having one side at the epiphyseal cartilage and including about one-fourth of the diameter of the cartilage, and another side at the outer surface of the bone and extending for a distance of about 1.5–2 mm. in which necrosis is marked and bacteria numerous. There is also a smaller necrotic region about the middle of the epiphyseal cartilage and including the bone on either side for a short distance. There is no inflammatory reaction about either of these regions. Organisms are present in practically all parts of the cancellous bone of the diaphysis and for a considerable distance from the epiphyseal cartilage in the epiphysis, also in many of the vessels of the compact bone. There are lesions of the bone marrow having an irregular arrangement of a deeply red-stained substance, bacteria and cells in which there is much nuclear fragmentation. In one such small lesion there is running through it a deep red-stained portion having the outline of a vessel and on either side of it are deeply stained cells, polymorphonuclear leukocytes and mononuclear cells. Bacteria are present both in the red-stained portion and among the cells surrounding it. At certain points in the marrow, usually next to the bone, there are rather large spaces, 0.5 mm. in length by about one-third that width, filled with bacteria alone and surrounded with marrow and bone that stain well. The lesions in the periosteum extend for a distance of 1–2 mm. along the diaphysis and for a much shorter distance along the epiphysis. Sometimes a single lesion extends along the periosteum for a distance of 1–2 mm. At other times there are a number of small

lesions separated by short intervals. They are of the same general character as those described in the bone marrow.

*Changes in a rabbit dying two days after a single intravenous injection of the 24-hour growth on one blood agar slant of a streptococcus (211) isolated from a case of "streptococcus septicemia" in the Chicago milk epidemic of 1912.*—Sections of the two carpal joints were examined. Streptococci are present in the blood vessels in all parts of the sections. There is a moderate amount of exudate in the joint cavities and in it are bacteria and a few cells. The synovial membrane and articular cartilages do not appear to be injured. There is extensive necrosis of the bone and bone marrow in the long bones, especially in the diaphyses, and to a less extent in the epiphyses and carpal bones. There is complete necrosis of portions of the diaphysis bordering on the epiphyseal cartilage, and a large number of organisms are present in these locations. Also in places, large masses of organisms separate the periosteum from the underlying bone. Where the bone marrow is not so greatly altered the vessels are filled with cocci. There is no reaction in any of the tissues.

*Changes in a rabbit dying two days after a single intravenous injection of the 24-hour growth on one blood agar slant of a streptococcus (213) from peritoneal fluid in a case of peritonitis secondary to sore throat.*—The changes found in the joints of this rabbit are quite similar to those found in the joints of the preceding rabbit.

*Changes in a rabbit dying two days after a single injection of the 24-hour growth from one blood agar slant of a streptococcus (214) from suppurating lymph glands secondary to sore throat.*—Sections from one carpal joint and one metacarpophalangeal joint were examined. The joint cavity of the metacarpophalangeal joint is not involved, but the cavities of the carpal joint contain a rather large amount of exudate in which are a large number of bacteria. Cells are not very numerous and consist of polymorphonuclear leukocytes and mononuclear cells which stain fairly well. The inner layer of cells of the synovial membrane is quite well preserved except in the recesses of the cavities where it is often necrotic. The blood vessels of the synovial membrane are distended with blood. There are many small collections of bacteria, some occupying the lumen of vessels with no inflammatory reaction about them. There are a few small necrotic regions containing scattered chains of cocci and surrounded by cells in which there is much nuclear fragmentation. There is also a considerable number of large mononuclear cells in the connective tissue of the plica synovialis especially near the blood vessels. There are a few necrotic places in the articular cartilages, one extending nearly the entire depth of the cartilage, otherwise there are no changes. In sections of the first phalanx of one of the toes, 0.5 mm. below the epiphyseal cartilage, there is a lesion in the bone marrow about 0.75 mm. square and occupying about one-fourth the diameter of the bone at this point. On three sides there is a zone of infiltration varying from one-tenth to one-sixth the diameter of the lesion, quite sharply demarcating it from the marrow. The fourth side, that nearest the epiphyseal cartilage, borders on necrotic cancellous bone and here, there is only a slight infiltration of cells. The infiltrating cells are mononuclear and polymorphonuclear leukocytes with a considerable number of eosinophils. The center of this lesion is occupied by cocci, arranged in long chains quite evenly distributed and not crowded. Neither necrotic material nor cells are present. The cancellous bone and the bone marrow near the epiphyseal cartilage are almost completely necrotic and the compact bone on one side is extensively involved for a distance of 2.5 mm. from the epiphyseal cartilage. The blood vessels contain numerous bacteria and the bone surrounding them is necrotic.

The periosteum covering this portion of the bone contains a lesion extending lengthwise of the bone for 2 mm. There is no reaction about any of the last mentioned lesions. A similar condition is present in the other long bones included in the sections. No lesions were observed in the bone marrow of the epiphysis or of the carpal bones.

*Changes in a rabbit dying two days after a single intravenous injection of the 24-hour growth on one blood agar slant of a hemolytic streptococcus (200) from the crypts of normal tonsils.*—Sections of the right carpal and left tarsal joints were examined. The cavities of both are filled with a very abundant exudate consisting of a granular, lightly hematoxylin stained material, cellular debris and organisms. The articular cartilages are very largely necrotic and in places there are erosions which extend entirely through them and communicate with abscesses in the bone marrow. The synovial membrane is also necrotic and the necrosis also extends for a considerable distance into the adipose tissue of the plica synovialis and the connective tissue immediately about the cavities. Separating the necrotic portions from the more normal tissues in certain places is a zone of cellular infiltration in which karyolysis is marked. Where the sections have been made across the prolongation of the synovial membrane laterally, the appearances obtained are those of an abscess. The tissues surrounding the joint cavities laterally are very edematous. There is a slight increase in the number of polymorphonuclear leukocytes and an increase in the mononuclear cells especially about blood vessels. In the tarsal joint the larger number of these cells are of the smaller more deeply staining type, occurring in groups of 20 to 50 cells or scattered more or less evenly through the tissue; in the carpal joint the large endothelial cells predominate. There are small regions of necrosis at various points in these tissues, often occurring in connection with blood vessels. Sometimes the entire vessel is included in the necrosis, sometimes there is a narrow band of necrosis surrounding a vessel or group of vessels. The endothelium is usually swollen and the vessel wall infiltrated with mononuclear cells and polymorphonuclear leukocytes. Lesions of the bone marrow are fairly numerous in the carpal bones especially near the articular cartilages. Most of them are small, well defined, necrotic regions in which there is infiltration of mononuclear cells and leukocytes. Bacteria are not always found. Others consist simply of collections of small, deeply stained, mononuclear cells. No lesions are found in the epiphyseal cartilages or the bone marrow near them. Some of the tendon sheaths contain an exudate similar to that found in the joint cavities, accompanied by necrosis of the tendon sheath, and an infiltration of the surrounding connective tissue with mononuclear cells and polymorphonuclear leukocytes.

*Changes in a rabbit killed four days after a single injection of the 24-hour growth from one blood agar slant of a streptococcus (242) isolated from the maxillary sinus.*—This rabbit was killed four days after injection. Sections from both carpal joints were examined. The joint cavities contain an abundant exudate consisting of a granular pink-stained material irregularly distributed, polymorphonuclear leukocytes, mononuclear cells and very large numbers of bacteria. The synovial membrane is quite generally necrotic near its attachment to the cartilages but in other places especially covering the plicae it is very well preserved. There are a few small patches of superficial necrosis in some of the articular cartilages. There are no lesions in the bone marrow of the carpal bones. In both radius and ulna of the same leg, there is in the diaphysis at one side, a triangular shaped piece of bone in which necrosis is complete. One side of the triangle lies along the epiphyseal cartilage and in the larger of these lesions extends about 2 mm. or nearly half the diameter of the bone. Another side lies

along the edge of the bone and is about 4 mm. in length. The periosteum is included in this lesion. Bacteria are very numerous in the marrow spaces and periosteum. There is no reaction about the lesion. In sections through the other carpal joint there is in one of the long bones necrosis of the bone and bone marrow of the epiphysis bordering on the epiphyseal cartilage for about four-fifths of its diameter and extending about one-half of one millimeter in the direction of the joint. Lesions similar to those in the periosteum are found in some of the tendon sheaths. Necrosis is marked and bacteria are numerous.

*Changes in a rabbit killed five days after a single intravenous injection of the 24-hour growth on two blood agar slants of a hemolytic streptococcus (192) isolated from the tonsils of a child five years old giving a history of tonsillitis associated with joint involvement eight months previous.*—Microscopic preparations of both carpal joints were examined. The cavities of the right joint contain a finely granular, lightly hematoxylin stained material, which probably represents fluid before it was fixed, and in it are bacteria and cells, many of which are disintegrating. There is extensive, sometimes complete necrosis of the synovial membrane, and the plica synovialis, and also the connective tissue immediately adjoining them. The articular cartilages are largely necrotic, and erosions extend entirely through them to the bone marrow. There is edema of the tissues surrounding the joint laterally, and an infiltration of mononuclear cells and leukocytes which is especially conspicuous about the blood vessels. Scattered through these tissues are many necrotic regions of varying size about some of which there is a cellular infiltration with much nuclear fragmentation. Occasionally the lumen of a vessel is found filled with bacteria and cells, chiefly mononuclears accompanied by little necrosis. One of the carpal bones is almost entirely necrotic; in others there is little necrosis of the bone, but small abscesses are numerous in the bone marrow bordering on the articular cartilages. There is also an occasional abscess in the bone marrow of the epiphyses of the long bones next to the articular cartilages or in the bone marrow of the diaphyses. There is an involvement of the tendon sheaths about the joint, which is quite similar to that of the joint cavities.

The process is accompanied by much less necrosis in the left carpal joint. The joint exudate is less abundant and contains fewer bacteria and more leukocytes, and there is less disintegration of cells. The synovial membrane is necrotic in places, especially in the recesses of the cavities. The adjoining connective tissue for a short distance is usually included in the necrosis. There is a moderate increase in the number of polymorphonuclear leukocytes in the surrounding connective tissue, especially near the joint cavities and about some of the blood vessels. Most conspicuous, however, is the infiltration of mononuclear cells around blood vessels (Fig. 2) in the connective tissue about the joints and tendon sheaths. This infiltration or proliferation of cells is sometimes outside the vessel wall on one side or, entirely surrounding it at other times, it is within the vessel wall (Fig. 3) beneath the endothelium, which is pushed forward into the lumen partially closing the latter. Some of the collections consist entirely of the large endothelioid cells, others of a combination of these with the smaller mononuclear cells and leukocytes. The tendon sheaths are infiltrated with mononuclear cells and leukocytes. Destruction is not extensive. In one tendon sheath there are alternate layers of cells staining blue and a deeply red-stained material containing a few polymorphonuclear leukocytes, making in all five layers, three blue and two red. The cells are mostly large mononuclears and polymorphonuclear leukocytes. There are a few superficial erosions of the articular cartilages but



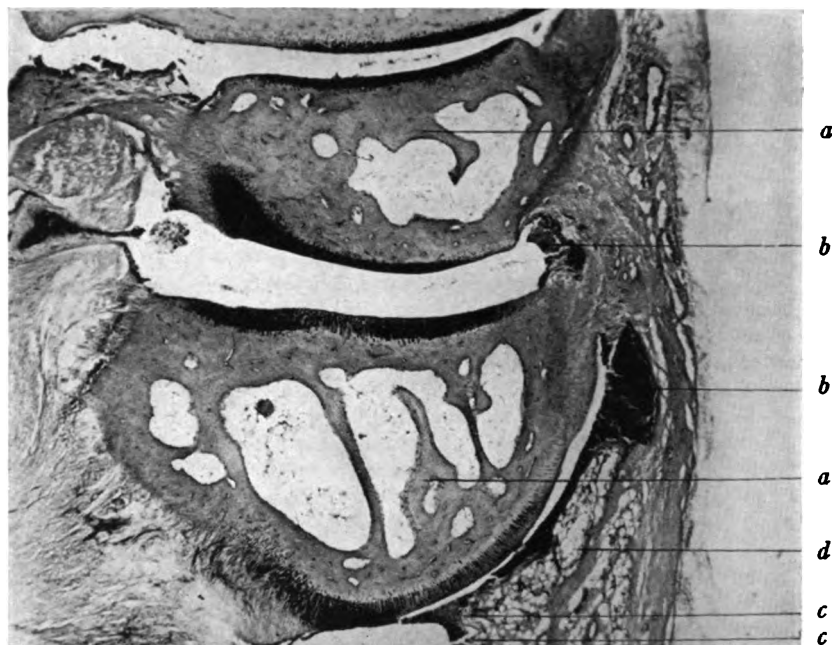


FIG. 1.—Exudate in the wrist joint cavities of a rabbit killed 24 hours after injection of streptococcus 236.

- |                                      |   |
|--------------------------------------|---|
| <i>a.</i> Carpal bones.              | <i>c.</i> Synovial fringes.                       |
| <i>b.</i> Exudate in joint recesses. | <i>d.</i> Adipose tissue in the plica synovialis. |

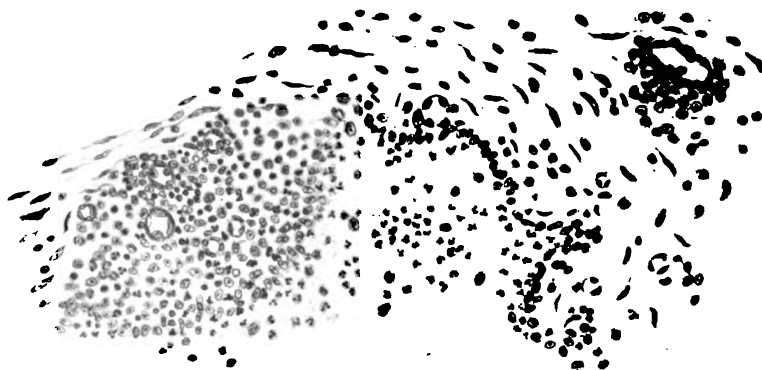


FIG. 2.—Small nodule adjacent to the synovial membrane, collection of mononuclear cells on one side of a small blood vessel and exudate in the joint cavity of a rabbit killed five days after injection of streptococcus 192.

no lesions of the epiphyseal cartilages, bone, or bone marrow were observed in this joint.

*Changes in a rabbit dying seven days after a single injection of the 24-hour growth of a streptococcus (218) isolated from the sero-fibrinous exudate of pleurisy following sore throat.*—The cavities of the ankle joint contain an exudate rich in cells which are largely polymorphonuclear leukocytes, and a few mononuclear cells with very little nuclear fragmentation or other evidence of necrosis, and many capsulated streptococci. The cells seem to be held together by a granular material which was formerly a fluid present in the joint cavity. In places this material is in the form of a very fine meshwork staining red in hematoxylin and eosin preparations, and resembling fibrin. The articular cartilages and synovial membrane appear to be very little injured except where the exudate is thickest next to the fringes that project into the cavity from in

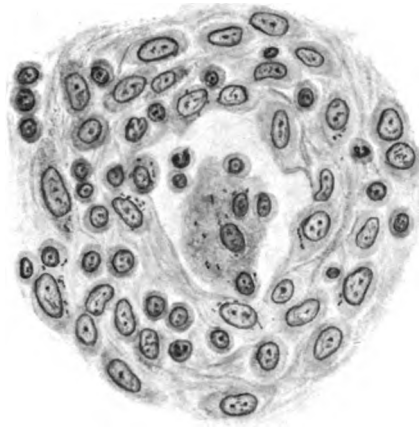


FIG. 3.—Collection of large and small mononuclear cells about a blood vessel containing a clot. Streptococci in the clot and in some of the cells. From a rabbit killed five days after injection of streptococcus 192.

front. Here there is a diffuse infiltration with polymorphonuclear leukocytes and destruction of the surface covering of synovial membrane. The tendon sheaths contain bacteria and are infiltrated with leukocytes and mononuclear cells with very little necrosis. There is a triangular lesion (Fig. 4) in the anterior portion of the diaphysis of the tibia, the sides being 1.5, 1, and 1 mm. respectively. It is bounded on one side by the epiphyseal cartilage, on another by the periosteum and on the third by the bone and bone marrow of the shaft. The bone and bone marrow are completely necrotic, the spaces being filled with bacteria and there is no infiltration of cells except along the side next to the epiphyseal cartilage. Hemorrhages are present in the bone marrow of the shaft 1.5 cm. from the joint surface. These are 1-1.5 mm. in diameter and occupy about two-thirds of the diameter of the shaft at that point. Sections of two toe joints were examined (Fig. 5), and in addition to the changes found

in the ankle joint, there are several small lesions in the bone marrow about 1.5 cm. proximal to the epiphyseal cartilage and located along the edge of the compact bone. These lesions are well defined and the largest is about 0.5 mm. in its longest dimension. There is an infiltration of mononuclear cells in the outer portions of the lesions and in the center are bacteria and much nuclear fragmentation. There is an infarct in the diaphysis of a bone of one of the toes quite similar to that described in the tibia. The epiphyseal cartilage in one of the bones of the other toe is completely dissected away from the cancellous bone of the diaphysis by a cellular exudate.

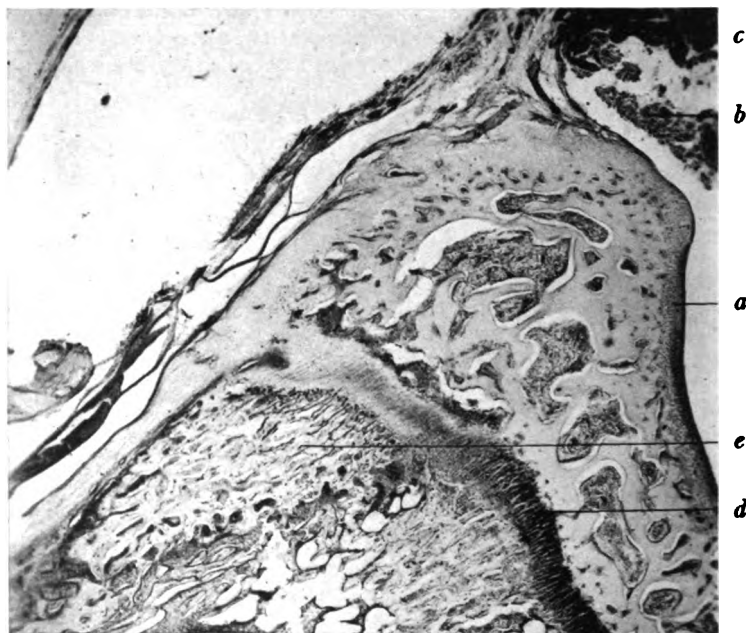


FIG. 4.—Infarct in the diaphysis of the tibia of a rabbit dying seven days after inoculation with streptococcus 218 from the sero-fibrinous exudate of pleurisy following sore throat.

- |                             |                          |
|-----------------------------|--------------------------|
| a. Articular cartilage.     | d. Epiphyseal cartilage. |
| b. Exudate in joint cavity. | e. Infarct.              |
| c. Synovial fringes.        |                          |

*Changes in a rabbit dying 11 days after a single injection of the 24-hour growth on one blood agar slant of a streptococcus (217) isolated from suppurating lymph glands following sore throat.*—Microscopic preparations of both carpal joints were examined. The cavities of these joints contain an abundant, finely granular, lightly hematoxylin stained exudate in which are a few cells, many bacteria, and scattered patches of eosin staining material. Fibrin was demonstrated in this exudate. The synovial membrane and plicae synovialis are completely destroyed and the necrosis extends more or less into the tissues about the recesses of the cavities and from these points lengthwise along the outer margin of the bones, in some cases as far as the epiphyseal cartilage. The

tissues surrounding these necrotic regions are edematous and densely infiltrated with mononuclear cells chiefly of the smaller variety. The blood vessels also contain many more than the normal number of mononuclear cells. Polymorphonuclear leukocytes are not greatly increased in numbers. Small foci of necrosis occur in these tissues in various places, and within and also about them as also in the tissue immediately surrounding the larger necrotic regions before described, there is a large amount of nuclear

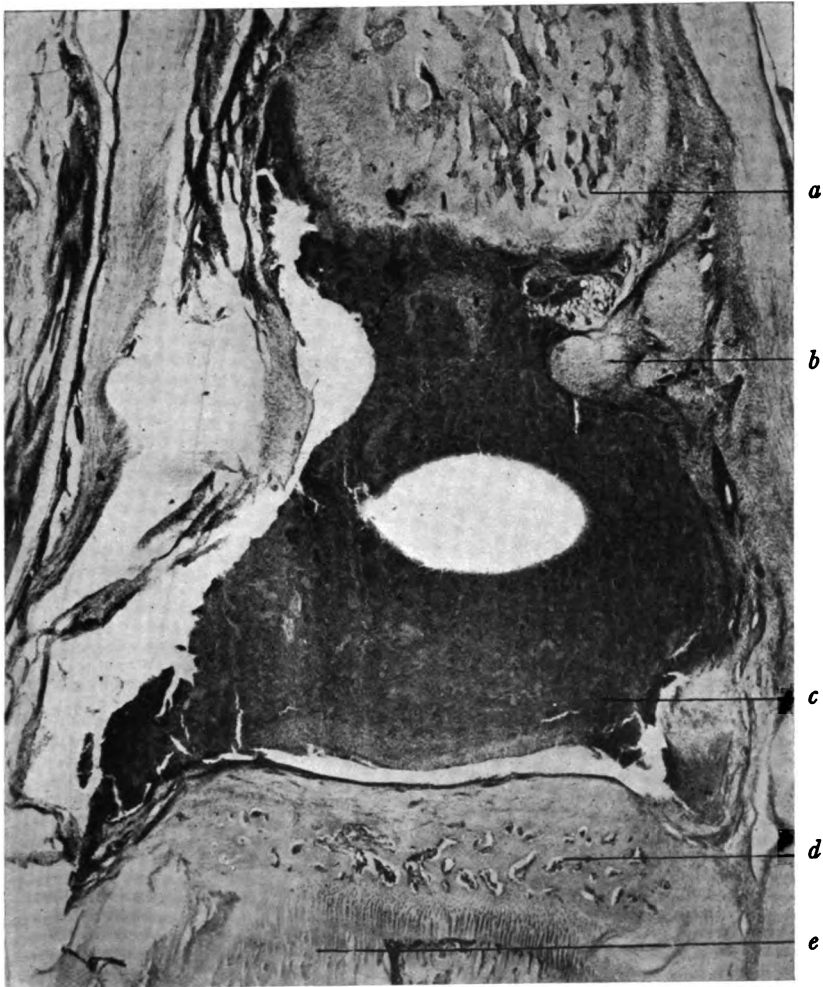


FIG. 5.—Large exudate in the metacarpo-phalangeal joint of rabbit dying seven days after inoculation of streptococcus 218 from the sero-fibrinous exudate of pleurisy following sore throat.

- |                                       |                     |
|---------------------------------------|---------------------|
| a. First phalanx of the toe.          | d. Metacarpal bone. |
| b. Plica synovialis.                  | e. Infarct.         |
| c. Large exudate in the joint cavity. |                     |

fragmentation. The cavities of the other joint contain a rather scanty exudate in which cells are quite numerous, some containing organisms but with little evidence of necrosis. There is very little injury of the synovial membrane except in a few places in the recesses of the cavities and some of the synovial fringes. There are a few regions of superficial necrosis in the articular cartilages. In the epiphysis of the radius just distal to the epiphyseal cartilage there is necrosis of the bone and bone marrow extending through nearly the entire diameter of the bone at that point and for about 0.5 mm. distalward. The epiphysis (Fig. 6) is practically dissected from the epiphyseal cartilage by a cellular exudate. In one place necrosis extends through the epiphyseal

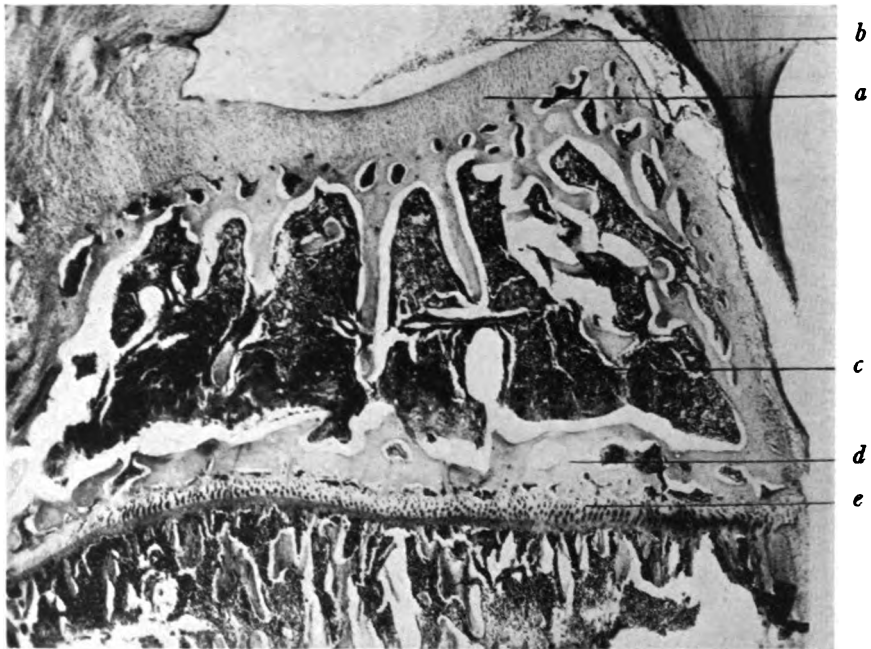


FIG. 6.—Partial necrosis of the epiphysis and epiphyseal cartilages of the radius of a rabbit dying 11 days after inoculation of streptococcus 217 from suppurating lymph glands secondary to sore throat.

- |  |                          |
|--|--------------------------|
| a. Articular cartilage.                                    | d. Necrotic bone.        |
| b. Exudate.  | e. Epiphyseal cartilage. |
| c. Cellular exudate in the marrow spaces of the epiphysis. |                          |

cartilage. There is some nuclear fragmentation of the cells of the bone marrow bordering on the lesion but the lesion is not sharply defined. There is also an infarct in the diaphysis of the radius resembling those described in bones of other joints.

*Changes in a rabbit killed 13 days after a single injection of a streptococcus (236) on a single blood agar slant.*—Sections of the carpal, elbow, and knee joints were examined. The cavities of the carpal joint contain an abundant necrotic exudate and numerous bacteria. The synovial membrane is absent and the plicae synovialis are necrotic. In the connective tissue about the joint there is the usual mononuclear infiltration and

scattered foci of necrosis. The articular cartilages are necrotic for the greater part of their depth and there are many small necrotic regions in the marrow of the carpal bones near the cartilages. The cavity and synovial membrane of the elbow joint appear normal. There are a few collections of mononuclear cells about small blood vessels in the connective tissues surrounding the joint cavity laterally. In the diaphysis of the ulna about 1.5 mm. from the proximal end of the bone, there is a small region of necrosis in the compact bone. The blood vessels in this portion of the bone are filled with a granular eosin stained material and many cocci. In the cancellous bone of the diaphysis next to the epiphyseal cartilage there is an infarct about 1.5 mm. in its longest dimension, that lies along the epiphyseal cartilage, and about 2 or 2.5 mm. proximal, and in the middle of the bone marrow there is a partially necrotic cell exudate. The cavity of the knee joint is distended with a necrotic exudate in which are large numbers of organisms. The synovial membrane is entirely absent and the connective tissue surrounding the cavity is necrotic for a variable distance from the margin. In one place the necrosis extends entirely through the tissues covering the joint anteriorly for a distance of 2 mm. There is a mononuclear infiltration of the connective tissue and muscle surrounding the joint cavity. The articular cartilages are necrotic for from one-half to two-thirds their depth, but no erosions are observed. No lesions of the bone or bone marrow are found. The tendon sheaths about the joint contain an exudate similar to that in the joint cavity and there is extensive necrosis of the surrounding connective tissues. The infiltrating cells are largely small mononuclears with comparatively few leukocytes and the infiltration is most marked about blood vessels.

*Changes in a rabbit killed two months and nine days after intravenous inoculation of the growth on one blood agar slant of a hemolytic streptococcus (256) isolated from the tonsils of a man aged 42 years, suffering from typical chronic arthritis of about one year's duration. These attacks of febrile exacerbation occurring every four to six weeks were accompanied by swelling of the wrist, shoulder, ankle and knee joints. The heart and kidneys were normal. The tonsils were moderately enlarged and not acutely inflamed. A practically pure growth of hemolytic streptococcus was obtained from plate cultures made from the crypts of both tonsils.*—Sections of the shoulder joint, the only joint involved, were examined. The inflammatory process in this joint differs considerably from that found in earlier stages, in the relatively great number of cells corresponding to the large mononuclear leukocytes, cells called by some, "endothelial," and markedly phagocytic. The joint cavity contains a moderate amount of exudate consisting of necrotic material and numerous cells, many partially disintegrated. These are chiefly polymorphonuclear leukocytes with a few mononuclear cells, some of which are very large. The synovial membrane is absent, and about the margins of the cavity ordinarily covered by it, is a heavily infiltrated region 0.5–1 mm. in thickness and necrotic near the cavity. The infiltrating cells are for the most part mononuclear cells with polymorphonuclear leukocytes scattered throughout and a few multinucleated giant cells in places. The mononuclear cells, both the large and small variety, are about equally numerous, the large cells more abundant close to the cavity and the small cells more abundant distant from the cavity. The large cells vary greatly in size and shape, are lightly stained with pale, round or oval nuclei, which are usually eccentrically placed and possess a large amount of cytoplasm, which is often loaded with detritus, pigment or bacteria. The cells containing pigment occur in groups and are usually located at some distance from the cavity. The small cells referred to are lymphocytes and plasma cells. At

the peripheries of this infiltrated region, they are aggregated into rounded masses resembling lymph nodes. In places this inflammation has extended to tissues surrounding the joint laterally; about many of the blood vessels are rings of small mononuclear cells varying somewhat in width, with the walls of many infiltrated with the same kind of cells. There are also in one place a few hemorrhages 0.5 mm. in their largest diameters. The articular cartilages are necrotic or entirely absent, and the bone thus exposed is also necrotic near the surface. For a distance of 5-6 mm. from the edge of the joint, the articular cartilage on the head of the humerus is either



FIG. 7.—Part of the joint cavity from a rabbit killed two months and nine days after injection of streptococcus 256.

- a. Exudate.
- b. Granulation tissue which has grown over the articular cartilage.
- c. Necrotic articular cartilage.

entirely absent or represented by small, scattered, thin layers of granulation tissue (Fig. 7), which in places is very well organized resembling an old scar, and in other places less so. This granulation tissue, where it is well organized and at other points, is directly continuous with the loose and edematous tissue of the marrow cavities of the cancellous bone. It is about 0.5 mm. in depth at its thickest point. There are in the bone marrow spaces underlying the articular surfaces, numerous collections of mononuclear cells and many osteoblasts. This joint differs also from the other joints in the irregularities found on the surface of the humerus. The outer lateral surface of the humerus just below the head for a distance of 1 cm. has an irregularly scalloped

appearance. These irregularities are elevated 1-1.5 mm. above the general level of the bone and constitute small but definite exostoses (Fig. 8). Around the outer edge,



FIG. 8.—Outer lateral surface of the humerus near the head of the bone, from a rabbit killed two months and nine days after inoculation with streptococcus 256.

*a.* Exostoses.

*b.* Nodule.

are large numbers of giant cells and internal to these are numerous small, newly formed marrow spaces and bone trabeculae. Still farther from the outer surface of the bone and near the newly formed bone are large numbers of osteoblasts.



Changes in a rabbit killed 24 hours after intravenous injection of the 24-hour growth on blood agar slants of "*Streptococcus viridans*" obtained from the heart valves of a man who long had suffered with endocarditis. The same streptococcus was found during life in the tonsils. Other injections had been given over a period of three months at intervals of 4, 8, 7, 2, 14, 16, and 12 days each. The first two were of the 24-hour growth on one blood agar slant of "*Streptococcus mucosus*" (28) isolated from the middle ear. Each of the remaining injections were of the 24-hour growth on five blood agar slants of the "*Streptococcus viridans*" previously mentioned.—This rabbit received eight injections, the last



FIG. 9.—Exudate and tissue reaction with a nodule containing giant cells from the elbow joint of a rabbit killed four months after inoculation with streptococcus 34 from the Boston epidemic of sore throat (Dr. Fabyan).

- a. Exudate in the joint cavity.
- b. Collection of mononuclear cells.
- c. Nodule composed largely of giant cells.

one being given 24 hours before it was killed. The joint cavities contain a small amount of exudate consisting of a granular eosin stained material, polymorphonuclear leukocytes, and a few streptococci. This exudate is found in larger or smaller masses lying close against the synovial membrane. The cocci usually occur in pairs, sometimes in short chains. The capillaries along the edge of the joint cavities are filled with blood and occasionally organisms are found in them. There is an increase in the number of polymorphonuclear leukocytes present in the vessels and tissues surrounding the joints. At various points along the synovial membrane of some of the joint

cavities and about small blood vessels in the plicae synovialis, there are collections of mononuclear cells with a few polymorphonuclear leukocytes. The mononuclear cells are chiefly of the smaller type. A considerable number of plasma cells are also present among them. In a few places the synovial fringes are necrotic. The surfaces of the articular cartilages are roughened and there are small regions of necrosis, some of them 0.5 mm. in length and often extending through the cartilage. In the diaphysis of the radius at a distance from the epiphyseal cartilage varying from 1.5 on one side to 0.75 mm. on the other is a region about 2 mm. wide in most places but extending about 1 cm. in a proximal direction along one side, in which osteoid tissue is abundant and osteoblasts numerous with only small patches of bone present here and there. This doubtless represents a healing infarct.

*Changes in a rabbit killed four months after a single intravenous injection of the 24-hour growth on three blood agar slants of a streptococcus (34) obtained from Dr. Fabyan of Boston.*—Sections of the left elbow and right tarsal joints were examined. In

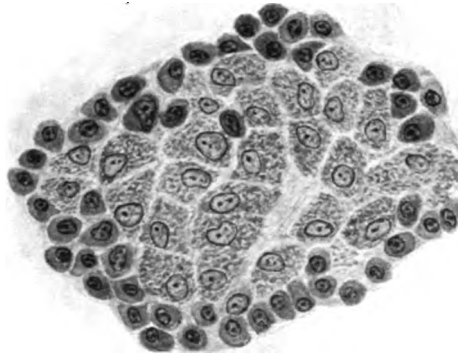


FIG. 10.—A group of large pigment-containing cells in the region of infiltration surrounding the elbow joint of a rabbit killed four months after injection of streptococcus 34.

sections of the elbow joint the lateral portions of the cavity and the boundaries have the appearance of an abscess cavity and wall. The cavity contains blood with more than the ordinary number of large mononuclear cells. About the cavity is a well defined wall of cellular infiltration (Fig. 9) varying in width from 0.5–1 mm. with the synovial membrane absent except in a few places. The cells composing the wall are small deeply stained mononuclear cells, large endothelioid cells, large, very lightly stained cells with small vesicular nuclei, and multinucleated giant cells. The small mononuclear cells are most numerous and about half of them are plasma cells which are scattered more or less uniformly through the infiltrated region; the other small cells, although present in all parts of this region, have a tendency to arrange themselves about blood vessels and in groups of varying size. The large endothelioid cells are unevenly distributed and many of them contain pigment. In some places they are particularly abundant about the border of the cavity; in other places they occur in patches, some containing three or four, others 50 or more cells. The pigment-containing cells (Fig. 10) are most numerous in the parts more distant from the

margin of the cavity. The large, very lightly-stained cells occur in groups near the outer edge of the lesion and especially in those parts which approach nearest the external surface of the leg. Groups of these cells are also present in the tissues between the infiltrated zone and the surface. The giant cells (Fig. 11) contain 2 to 10 or 12 nuclei which are usually arranged about a central portion which contains red corpuscles, pigment, or a granular, pink-stained substance. They are arranged in groups, sometimes forming distinct nodules which are located near the margin of the cavity or at the outer part of the wall. The bone, except for a narrow strip bordering on the cavity which is necrotic, and the bone marrow, appear unchanged.

The contents of the left tarsal joint appear normal. The plica synovialis seems to be unchanged, being made up largely of fat cells. The surfaces of the articular



FIG. 11.—A nodule of multinucleated giant cells in the infiltrated region surrounding the elbow joint of a rabbit killed four months after injection of streptococcus.

cartilages (Fig. 12) are very irregular, due to partial or entire erosion of the cartilage and to outgrowths of cartilage into the cavity. There are also rather large necrotic regions in the cartilages which include the underlying bone for a short distance. In many places the cartilage cells are arranged in a very irregular manner, deeply-stained cells occurring in clumps singly or in groups and separated by rather wide spaces of cartilage containing no cells. In one place where the cartilage has been eroded for its entire depth, the space is filled in with a very vascular connective tissue which spreads out over the surface of the surrounding cartilage for a distance of 0.5 mm. There are collections of deeply staining mononuclear cells in many of the bone marrow spaces beneath the articular cartilages which have been injured. In one of the tarsal bones there are three small regions closely associated and somewhat rounded in shape with centers occupied by a lightly hematoxylin stained, finely granular substance in which are a few nuclei. About these regions is fibrous tissue arranged in a concentric manner.

*Changes in a rabbit killed four months after a single intravenous injection of the 24-hour growth on two blood agar slants of a "Streptococcus viridans" (248) isolated from the crypts of the tonsils in a case presenting no evidences of endocarditis or arthritis.*—Microscopic preparations of the left carpal and one tarsal joint were examined. The lesions found in the tarsal joint are fewer but of the same character as those found in the carpal joint. The synovial membrane and plicae synovialis appear unchanged. The fibro-cartilaginous portion and to some extent the adjoining articular cartilage are partially necrotic and in places have a shredded or coarsely fringed appearance with

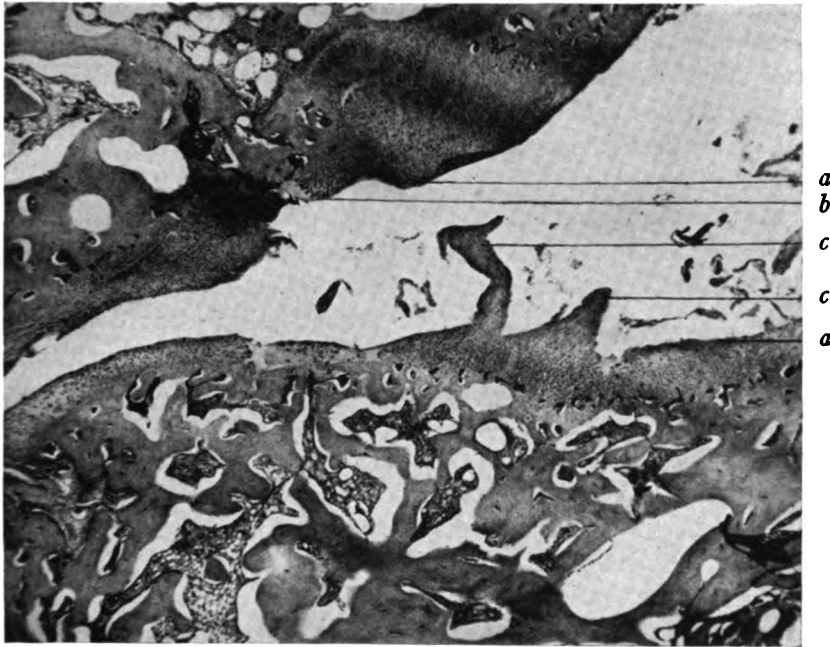


FIG. 12.—Ecchondroses of the cartilages of the tarsal joint of a rabbit killed four months after injection of streptococcus 34.

a. Articular cartilages.

b. Erosion of the cartilage.

c. Ecchondroses.

groups of cells arranged at intervals along the shreds or fringes. The surfaces of the articular cartilages are rather uneven, and there are small regions of necrosis, some extending nearly through the cartilage and a few shallow erosions. In places the cartilage cells are small and irregularly arranged. No lesions of the bone or bone marrow were observed.

The differences in the inflammatory process in the joints of the 17 rabbits of this series studied at periods varying from two hours to four months (136 days) are only such as are consistent with the

varying phases of a single inflammatory process, nor are there any striking differences in the reaction produced by the various kinds of streptococci employed. The changes in the joints of rabbits inoculated with hemolytic streptococci were studied after periods of 4, 5, and 70 days; those produced by *Streptococcus mucosus* at the end of 95 days, and the joint changes caused by *Streptococcus viridans* at the end of 136 days. The largest number of joints studied were from rabbits inoculated with streptococci isolated from human lesions produced by the milk epidemic and the peculiarities of these streptococci on culture media have been described by Dr. Davis.<sup>1</sup> These animals died or were killed after periods of 2, 4, 10, and 24 hours and 2, 3, 4, 7, 11, and 13 days. It may be that had more joints been studied from rabbits inoculated with the forms of streptococci least used, e.g., *Streptococcus viridans* and *Streptococcus mucosus*, some variation in the inflammatory process would have been found. The alterations described depend upon the early and apparently simultaneous and independent localization of the bacteria in the joint cavity, in the tissues surrounding the blood vessels of the synovial membrane, plica synovialis, tendon sheaths and blood vessels of the periosteum and bone marrow near the epiphyseal cartilages. The rather extensive involvement of the peri- and parasynovial structures such as tendon sheaths, epiphyseal cartilages and bone marrow is perhaps more marked than has been described by the authors quoted in the literature reviewed. The nodular formations developing in the tissues about the joints are essentially similar to those described in the myocardium of some of these same animals<sup>2</sup> and constitute a feature of the healing or later stages of the inflammation produced not only by *Streptococcus viridans* but also by the streptococcus from the epidemic.

Of two points deserving special mention, one is the vulnerability of the triangular, loosely constructed fibro-areolar tissue projecting into the joint laterally, the so-called plica which contains varying amounts of adipose tissue. It may be that the spirally arranged blood vessels here<sup>3</sup> offer exceptional opportunities for the lodgment

<sup>1</sup> Jour. Am. Med. Assn., 1912, 58, p. 1852.

<sup>2</sup> Jour. Infect. Dis., 1912, 11, p. 243.

<sup>3</sup> L. Testut, Traité d'anatomie humaine, 6th ed., Paris, 1911, 1, p. 429.

of bacteria or are as much end vessels as those next to the epiphyseal cartilages. The other point concerns the influence on the character of the inflammatory process exerted by the peculiarities of joints in that they are at the same time free surfaces and closed cavities. Undoubtedly these features contribute in ways we do not fully understand to the results of infection, especially the absorption of inflammatory exudates and the extent to which healing is possible.

## INTERRELATIONS IN THE STREPTOCOCCUS GROUP WITH SPECIAL REFERENCE TO ANAPHY- LACTIC REACTIONS.\*

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Numerous methods for the differentiation of streptococci have been attempted by many workers with varying degrees of success. Various morphological and cultural details were used, especially by the earlier workers in this field. On the whole specific immune sera have not been satisfactory on account of the fact that this group is not highly sensitive to the various immune reactions. The acid agglutination method, used by Michaelis with success in connection with the typhoid-colon group, has recently been applied by Bergey<sup>1</sup> to the streptococcus group without definite results. Fermentation tests are undoubtedly of value, but on account of the tendency of the organisms to vary in their fermentative powers this method is not an absolutely reliable one for differential purposes.

The clinical manifestations of streptococcus infections are so varied that the idea that important differences must exist between the cocci constantly appeals to one. I became especially interested in this problem when studying the flora of the tonsillar crypts in cases of chronic renal, cardiac, and articular diseases. Though there was good reason to believe that the clinical condition was caused by the tonsillar infections in many instances, still the streptococci isolated from the various cases could not be differentiated.<sup>2</sup> Erysipelas, puerperal sepsis, septicemia, pyemia, various skin lesions, tonsillitis, etc., are only further illustrations of this point.

In the study of these organisms, it is undoubtedly true that emphasis has usually been placed on minute and often unimportant details which might serve to differentiate them, rather than on points which might serve to demonstrate their close relationship or, possibly their identity. Reference may here be made to my brief preliminary communication entitled "Relation of Varieties of

\* Received for publication March 5, 1913.

<sup>1</sup> *Jour. Med. Res.*, 1911, 27, p. 67.

<sup>2</sup> *Jour. Infect. Dis.*, 1912, 10, p. 148.

Streptococci with Especial Reference to Experimental Arthritis,"<sup>1</sup> which may serve as an introduction to the present paper.

The relations existing between members of the streptococcus group were there represented in a table which has since been amplified but not essentially altered by the addition of other data. This amplified table is published now together with some explanatory notes and also with some data bearing upon the possible transformation of one variety into another. In order to inquire farther into the relations of members of this group of organisms some interanaphylactic experiments have been made with streptococci from various sources the details of which follow.

In Table 1, the organisms have been arranged in order as determined by morphological, cultural, and experimental data. These are: (1) hemolysis on blood agar plates, (2) production of green colonies on blood agar plates, (3) capsule formation, (4) solubility in bile, (5) inulin fermentation, (6) experimental arthritis in rabbits, and (7) experimental endocarditis in rabbits. When the members of the streptopneumococcus group are arranged in the order given in the table, it will be seen that a more or less gradual transition occurs from one variety to another with reference to the properties enumerated. In general, the sign  $\approx$  indicates that the particular property in question may occur rarely or to a very slight degree and the number of + signs is intended to give a general idea of the intensity or the relative frequency, as the case may be, of the property.

TABLE 1.  
RELATIONS OF STREPTOCOCCI.

	Organisms	Hemolysis	Green Colonies on Blood Agar	Capsule	Solubility in Bile	Inulin Fermentation	Experimental Arthritis	Experimental Endocarditis
1	Str. hemolyticus.....	+++	o	o	o	o	+++	$\approx$
2	Str. epidemicus.....	++	o	+	$\approx$	+	+++	+
3	Str. mucosus.....	$\approx$	++	+++	+++	++	++	$\approx$
4	Str. pneumoniae.....	o	+++	+	++	++	$\approx$	+
5	Str. viridans.....	o	+++	o	o	+	$\approx$	+++

The first member or group in the series, *Streptococcus hemolyticus*, is the common streptococcus usually called *Streptococcus pyogenes*. Here are included the streptococci found in acute sore throats,

<sup>1</sup> Jour. Am. Med. Assn., 1912, 58, p. 1283.



septicemias, etc.; also the streptococci that commonly occur about the tonsils and throat in cases of arthritis, nephritis, etc. It should be stated that the term *Streptococcus pyogenes*, as commonly used, is a broad one and may perhaps with equal propriety be applied to the first three members of the series. No. 2 is the organism that was encountered in the epidemic of sore throat that prevailed in Chicago during the winter season of 1911-12. For convenience we refer to this organism as the epidemic streptococcus. No. 3 is the fairly well-defined organism, now commonly called *Streptococcus mucosus*, which was formerly known as *Streptococcus mucosus capsulatus*. *Streptococcus capsulatus* and *Pneumococcus mucosus* are other terms which have been applied to this organism. No. 4 in the series is the ordinary pneumococcus which for the sake of uniformity of nomenclature is referred to as *Streptococcus pneumoniae*, a term not infrequently used by authorities in connection with this germ. No. 5 is *Streptococcus viridans*, an organism to which especial attention was first called by Schottmüller and Rosenow. This organism is commonly found about the mouth and throat, and appears to be identical with the organism found in subacute or chronic infectious endocarditis as observed by Rosenow, Schottmüller, Libman, and others.

These organisms all appear to be fairly well defined and, as stated, arrange themselves in definite order in the table with respect to certain properties. As regards hemolysis, the first members are hemolytic; as we descend the series, this property vanishes. The production of green colonies on blood is not noted in the first members but occurs lower in the series.

Capsule formation is most marked in the middle member, namely *Streptococcus mucosus*, and diminishes in either direction, the members at the top and bottom of the series ordinarily not showing this property. Exceptions to this rule occur especially in the hemolytic streptococcus group, when capsules may appear as a result of growth in animals or animal fluids. The question then arises as to their possible transformation into the second or epidemic type.

In 1900 Neufeld<sup>1</sup> first called attention to the relative solubility

<sup>1</sup> *Ztschr. f. Hyg. u. Infektionskrankh.*, 1900, 34, p. 454.

in bile of this group of organisms, and his results have since been confirmed especially by Grixoni<sup>1</sup> and by Libman.<sup>2</sup> As a means of differentiating streptococci and pneumococci, Libman and Cellar<sup>3</sup> have called particular attention to this method. The results of various investigators including some data obtained by the writer on this point, indicate, as shown in the table, that practically all strains of *Streptococcus hemolyticus* are insoluble in rabbit's bile. *Streptococcus epidemicus* is insoluble, or only to a very slight extent soluble, in rabbit's bile. *Streptococcus mucosus* is highly soluble, and the pneumococcus is also soluble, but somewhat less so than *Streptococcus mucosus*. *Streptococcus viridans* or endocarditic coccus is insoluble. It will thus be seen that in a general way the solubility in bile runs parallel with capsule formation. Whether or not it is dependent upon capsule formation has not been definitely determined.

Sugar fermentation with the streptococcus group has on the whole not been trustworthy or satisfactory on account of inconsistency of results. The work of Walker<sup>4</sup> in this respect may be consulted. The fermentation of inulin has been used as a differential point between pneumococci and streptococci and is of value, though it cannot be considered entirely reliable. For example, Libman found that of 19 strains of pneumococci, all fermented inulin except two; of 12 strains of *Streptococcus mucosus*, all fermented inulin, and of 69 streptococci, all failed to ferment inulin except two and these lost this property after animal passage. Of *Streptococcus viridans* from cases of endocarditis, about one third ferment inulin. On account of the variation of this property the relative fermentative power of the various cocci can only be represented roughly in the table.

In a study of the pathogenic properties of various streptococci, I have found that experimental arthritis is readily produced in rabbits by the upper three varieties when the organisms are given intravenously.<sup>5</sup> It is rarely produced by strains of the last two organisms. In general the reverse is true concerning experimental

<sup>1</sup> *Riv. crit. di clin. Med.*, 1909, 10, 17.

<sup>2</sup> *Proc. Path. Soc.*, New York, 1908, 8, p. 40.

<sup>3</sup> *Am. Jour. Med. Sc.*, 1910, 140, p. 516.

<sup>4</sup> *Proc. Royal Soc. London*, 1911, Series B, 83, p. 541.

<sup>5</sup> *Jour. Am. Med. Assn.*, 1912, 58, p. 1852.

endocarditis in rabbits. In about 10 per cent of rabbits inoculated intravenously with hemolytic and epidemic streptococci, endocarditis results. Rosenow<sup>1</sup> has shown that experimental endocarditis may be produced in nearly every instance by inoculation with organisms of the viridans type.

It is to be noted that the transition from one organism to another is more or less gradual, a point which brings up the question of the possible transformation of one variety into another. The variability of bacteria is a subject which has been studied by many observers for years and many data concerning numerous varieties exist. For example, the transformation of a golden staphylococcus into the white variety is a well-known phenomenon. The loss of the property of hemolysis by strains of *B. coli* has been repeatedly observed. Ruediger<sup>2</sup> and also Anthony<sup>3</sup> have noted the occasional loss of hemolysis in certain strains of streptococci. The latter writer noted changes in hemolysing power in about five per cent of the organisms tested. Attention has already been called<sup>4</sup> to the transition of the epidemic streptococcus to the ordinary hemolytic streptococcus and vice versa. The strains vary markedly in this respect, some apparently retaining their properties with much greater tenacity than others.

That more extensive variations also take place at times and under certain conditions is undoubtedly true. I presume it is a fact that nearly every investigator working in bacteriology, especially with minute cultural details, can cite observations which at least suggest mutation.<sup>5</sup> A few instances which have come under my observation are here cited. Five guinea-pigs were each inoculated with one cubic centimeter of a 24-hour broth culture of a typical hemolytic streptococcus (No. 243), which several weeks previously had been isolated from the tonsils of a case of ordinary sore throat. The zone of hemolysis was wide and clear and without green color. There was no capsule and the growth on blood agar slants was not moist or spreading. Four of the guinea-pigs showed

<sup>1</sup> *Jour. Infect. Dis.*, 1909, 6, p. 245.

<sup>2</sup> *Jour. Infect. Dis.*, 1906, 3, p. 663.

<sup>3</sup> *Ibid.*, 1909, 6, p. 332.

<sup>4</sup> *Jour. Am. Med. Assn.*, 1912, 58, p. 1852; also Rosenow, *Jour. Infect. Dis.*, 1912, 11, p. 338.

<sup>5</sup> An excellent monograph including literature on this subject is by Hans Pringsheim: *Die Variabilität niederer Organismen*, Berlin, 1910.

little or no reaction and were alive and well at the end of three weeks. One of the animals died on the 10th day after the infection. A gelatinous turbid exudate was found in the peritoneum and in the pleural cavities. Smears of the exudate revealed many encapsulated diplococci and short chains. Cultures from the heart's blood and the exudates gave pure growths of an organism that has the properties of *Streptococcus mucosus*. The growth on blood agar is very moist, raised, and mucoid. The colonies are distinctly green and about them is a green zone without hemolysis or with only a slight trace appearing after two or three days. The organism grows scarcely at all on plain media or on sugar media. It is distinctly soluble in rabbit's bile but does not ferment inulin. At the present time, which is about one month after isolation, there is no tendency to revert to the hemolytic type. The growth is perhaps less viscid and spreading than when first isolated, but on blood plates the colonies are distinctly green and perhaps tend to resemble the pneumococcus more than the hemolytic or epidemic streptococci. It is highly virulent for animals and animal passage causes an increase in its mucoid properties. While the tendency culturally seems to be toward the pneumococcus type, morphologically it is not a pneumococcus. It should be stated that the original streptococcus, which in the first place was grown from a single plate colony, was plated later without finding any colonies of the mucosus type. It appeared to be in the original, therefore a pure strain.

Another instance of a transformation in the reverse order was noted as follows: An organism of the mucosus type was isolated from an exudate. The colonies were green and mucoid and without a hemolytic zone. This organism was injected into the vein of a rabbit and a few days later arthritis appeared in one of the hind legs of the animal. The joint became enlarged and distinct fluctuation was present. After a number of days the joint was aspirated and a small amount of a seropurulent exudate obtained. When plated, this yielded a pure growth of a streptococcus which was strongly hemolytic, and the colonies showed no green color and no marked mucoid or spreading growth.

The transformation of *Streptococcus viridans* into an organism

quite like the pneumococcus and the reverse process have been noted especially by Rosenow<sup>1</sup> in connection with his exhaustive work on the endocarditic cocci. He believes that *Streptococcus viridans* is a modified pneumococcus and by altering certain growth conditions, the one may acquire the properties of the other.

In order further to test the relations of members of the streptococcus group it was thought that possibly anaphylactic reactions which have been applied to other groups of bacteria might be of value here.

Kraus and Doerr<sup>2</sup> have shown that guinea-pigs sensitized to typhoid bacilli, while reacting to typhoid bacilli, would not react to such closely related organisms as the dysentery bacillus and the paratyphoid bacillus. Also guinea-pigs sensitized to the cholera vibrio would not react to the typhoid bacillus nor to some apparently closely related organisms (*Vibrio Nasik*). They point out that this method may be of value in determining the relationship of bacteria.

Guerrini<sup>3</sup> tested various bacterial nucleoproteids and concluded that anaphylaxis is specific for the "Bacterienart" from which the nucleoproteid was obtained. In his experiments he made inter-anaphylactic tests with *B. pestis*, *vib. cholerae*, and certain organ nucleoproteids but did not use proteids from such closely related organisms as did Kraus and Doerr.

Guinea-pigs were sensitized intraperitoneally with small amounts of cocci (one-half slant growths), which had been grown for 24 hours on human blood agar free from water of condensation and then suspended in salt solution. Washing of the bacteria was done in some instances but was found unnecessary. Furthermore Boehnke and Bierbaum<sup>4</sup> found that the peptone in the media plays no part in the sensitization with bacteria because the phenomenon is not altered by using peptone free media. This factor therefore could be disregarded. After an interval of from 14 to 26 days they were intoxicated, intracardially or by injections into the jugular vein, with the various organisms to be tested. The growth from two or three large slant tubes was used for intox-

<sup>1</sup> *Jour. Infect. Dis.*, 1910, 7, p. 411.

<sup>2</sup> *Wien. klin. Wchnschr.*, 1908, 21, p. 1008.

<sup>3</sup> *Ztschr. f. Immunitätsf. u. exp. Ther.*, 1912, 14, p. 70.

<sup>4</sup> *Centralbl. f. Bakteriol.*, 1 Orig., 1912, 65, p. 504.

ication and for culture media; horse blood agar was used in many instances though the human blood media, such as was used for sensitization, gave apparently the same results. The organisms in all instances were immediately injected after suspension in order to avoid the formation of anaphylatoxins.

The results are given in Table 2. In the first place organisms of the type of *Streptococcus hemolyticus* appear to interact freely. For example, a streptococcus (243) isolated from the tonsils in a case of acute follicular tonsillitis reacted with *Streptococcus hemolyticus* from a case of erysipelas. Streptococci isolated from ordinary sore throats react with cocci from throats of chronic arthritis as do also hemolytic streptococci causing otitis media. Furthermore organisms of the ordinary hemolytic variety react with organisms of the type we have called "epidemicus." In this group may be considered a streptococcus which was isolated from the udder of a cow suffering from mastitis. This organism after animal passage acquired the characteristics of the epidemic streptococcus. It reacted positively with a hemolytic streptococcus isolated from the throat of a case of ordinary tonsillitis. A strain of streptococcus known as the "grip" streptococcus was kindly sent to me by Dr. Seligmann of Berlin. This organism was isolated from a patient suffering from a streptococcus infection which appeared in epidemic form among children, the symptoms of which were a severe "grippe." The epidemic occurred in Rummelsburg, Germany, in 1911, and has been reported in detail by Müller and Seligmann.<sup>1</sup> The streptococcus has the characteristics of *Streptococcus epidemicus* after animal passage, though when first received it resembled more closely *Streptococcus hemolyticus*. Dr. Seligmann<sup>2</sup> called my attention to the fact that it had lost on artificial media many of its peculiarities. It reacts strongly positive in a guinea-pig sensitized with a typical hemolytic streptococcus (243), exactly as do the epidemic streptococci isolated here in Chicago. A strain of streptococcus, isolated during the milk epidemic in Boston in 1911, obtained from Dr. Fabyan of Boston, reacted with an animal sensitized with a typical hemolytic streptococcus. Also a streptococcus isolated from the Chicago epidemic reacted

<sup>1</sup> *Berl. klin. Wchschr.*, 1911, 48, p. 1636.

<sup>2</sup> Personal communication.

TABLE 2.  
INTERANAPHYLACTIC REACTIONS IN GUINEA-PIGS WITH STREPTOCOCCI.

	Sensitizing Organism	Intoxicating Organism	Interval between Inoculations	Result
1	Str. hemolyticus (225). Isolated from middle ear discharge.	Str. hemolyticus (225). 2½ slant growths intracardially.	17 days	Death in 4 minutes from typical anaphylactic shock. Lungs very pale and markedly distended.
2	Str. hemolyticus (256). Isolated from tonsillar crypts in a case of chronic arthritis which promptly recovered following extirpation of tonsils.	Str. hemolyticus (225). 2 slants intracardially.	17 days	Death in 3 minutes from violent anaphylactic shock. Lungs show characteristic distention.
3	Str. hemolyticus (256).	Str. hemolyticus (243). From tonsils in a case of follicular tonsillitis. Injected 2 slant growths.	21 days	Typical symptoms of anaphylaxis in 1 minute. Animal dead in 3 minutes. Lungs very emphysematous.
4	Str. hemolyticus (257). Isolated from tonsils in a case of chronic rheumatism.	Str. hemolyticus (256). 3 slants injected.	17 days	In a few minutes slight but definite symptoms of shock. Recovery.
5	Str. hemolyticus (243).	Str. from erysipelas. 3 slant tubes injected into jugular vein.	19 days	In 2 minutes symptoms appeared. Restlessness, defecation, hair raised about head, rubbing of nose, squeals at intervals. No definite spasms. Recovery.
6	Str. hemolyticus (243).	Str. "Madel." Isolated by Dr. Seligmann from case of grip epidemic in children in Germany. 2 slants injected intracardially.	17 days	In about 2 minutes distinct signs of shock. Recovery.
7	Str. "Madel."	Str. "Madel." 2 slant growths intracardially.	17 days	Slight but distinct symptoms of anaphylaxis in few minutes. Recovery.
8	Str. hemolyticus (229). Isolated from ear discharge.	Str. epidemicus (211). Isolated from spleen at autopsy.	22 days	Death in about 3 minutes from typical anaphylactic shock. Lungs very highly distended.
9	Str. hemolyticus (250). Isolated from tonsils in child suffering with slight heart lesion.	Str. epidemicus. Isolated by Dr. Fabyan during Boston epidemic. 4 slant tubes injected.	22 days	In 2 minutes distinct anaphylactic shock. Spasm very marked. Gradual recovery.
10	Str. hemolyticus (243).	Str. mucosus. Isolated from ear discharge. 2 slants injected into heart.	24 days	Immediate convulsions. Respirations very violent. At intervals severe spasms. Gradual improvement. Death after 12 hours. Lungs moderately distended.
11	Str. hemolyticus (243).	Str. pneumoniae. Isolated from pleural cavity at autopsy in case of lobar pneumonia. 3 slant tubes injected into jugular vein.	19 days	In about 4 minutes, spasms, rubbing of nose, heavy breathing, etc. After one hour animal still having spasms. Killed. Lungs very emphysematous. Hemorrhages into stomach and myocardium.
12	Str. hemolyticus (243).	Str. pneumoniae. Isolated from rusty sputum of case of lobar pneumonia. Injected into jugular vein.	26 days	After 3-4 minutes distinct spasms, very restless, heavy breathing, rubbing of face, etc. Gradually recovered. Two days later given Str. hemolyticus. No reaction. Animal killed in 15 minutes. Lungs show no emphysema.

TABLE 2.—Continued.

	Sensitizing Organism	Intoxicating Organism	Interval between Inoculations	Result
13	Str. epidemicus (214).	Str. pneumoniae (same as No. 11).	14 days	In a few minutes animal showed distinct symptoms. Rubbing of nose, defecation, jerky spasms, restlessness, etc. Recovery.
14	Str. pneumoniae. Isolated at autopsy.	Str. hemolyticus (243) into heart.	21 days	After about one minute violent spasms appeared with death in 4 minutes. Lungs highly emphysematous.
15	Str. epidemicus. Isolated from udder of cow suffering from mastitis.	Str. hemolyticus (243). Intracardial injection.	22 days	After a few minutes distinct symptoms. Hair on head erect, frequent defecation, scratching of nose, heavy breathing. Died in 20 minutes from hemorrhage into pericardium. Lungs distinctly emphysematous.
16	Str. epidemicus (strain from Boston epidemic).	Str. epidemicus (233). Isolated during Chicago epidemic.	22 days	In a few minutes signs of anaphylaxis. Restlessness, defecation, jerky spasms, rubbing of nose, etc. Recovery.
17	Str. epidemicus (233).	Str. viridans. Isolated from case of endocarditis.	26 days	No distinct signs of anaphylaxis. Had some circus movements. Died during night. Lungs showed questionable evidence of anaphylaxis.
18	Str. hemolyticus (243).	Str. viridans (endocarditis). 3 slant tubes injected into jugular vein.	19 days	Practically no symptoms. No spasms or restlessness. 5 days later injected intracardially 2 tubes Str. hemolyticus 243. Typical spasms with death after few minutes. Lungs emphysematous.
19	Str. hemolyticus (243).	Str. viridans (endocarditis). Intracardial injection.	24 days	No definite symptoms. Killed after 20 minutes. Lungs practically normal.
20	Str. hemolyticus (243).	Sta. albus. Isolated from skin. 2 slant cultures.	15 days	No symptoms.
21	Str. hemolyticus (243).	Sta. albus. Isolated from furuncle. 2 slant cultures.	15 days	No definite symptoms.
22	Str. hemolyticus (243).	Sta. aureus. Isolated from carbuncle. 2 slant cultures.	15 days	No definite symptoms.
23	Normal guinea-pig.	Injected with 2 tubes of Str. hemolyticus (243).	.....	No anaphylactic symptoms.
24	Normal guinea-pig.	Injected with 2 tubes of Str. epidemicus.	.....	No symptoms.
25	Normal guinea-pig.	Injected with 3 tubes of Str. viridans.	.....	No anaphylactic symptoms.

positively with a guinea-pig sensitized with the Boston streptococcus. Furthermore it was found that a typical *Streptococcus mucosus* reacted very definitely with an animal sensitized with the



hemolytic streptococcus and also with the epidemic streptococcus. Likewise the hemolytic streptococcus reacted in an animal sensitized with *Streptococcus pneumoniae*. Two strains of *Streptococcus pneumoniae* were tested, one of which was isolated from the heart's blood postmortem; the other was isolated in pure culture from the sputum of a case of lobar pneumonia.

*Streptococcus viridans* in my experiments failed to react with animals sensitized with the hemolytic streptococcus; or at least the reaction, if present at all, was very slight. Furthermore, two days later one of the animals reacted promptly to an intoxicating injection of the sensitizing streptococcus. *Streptococcus viridans* used in these experiments was isolated by blood culture from a case of chronic infective endocarditis.

Animals sensitized with *Streptococcus hemolyticus* were intoxicated with *Staphylococcus albus* from the skin, one from an abscess and a *Staphylococcus aureus* from a carbuncle. No reaction appeared in any instance.

The above results seem to indicate that there is a close relationship between various members of this group with the exception of the organisms of the viridans type. In the interpretation of these results I think caution should be used. It is undoubtedly true that the bacteria mentioned have anaphylactic factors in common, but I would not consider the data as excluding the possibility of the existence of certain specific elements in the various organisms such as Wells<sup>1</sup> has shown to occur in certain closely related vegetable proteins. In order to determine this point, more careful studies, especially with chemically purified bacterial proteins, will have to be made.

The results of the anaphylactic experiments agree in a general way with the data given in the first part of the paper. They tend to corroborate the idea brought out in Table 1 and with possibly one exception explain the apparently easy transition from one member to another. The failure of *Streptococcus viridans* to react was not expected. It may be that there is a gap existing between this organism and the other members of the series which might be bridged over by strains occupying intermediary positions.

<sup>1</sup> H. G. Wells, paper read before the Chicago Path. Soc., February 10, 1913.

I used an endocarditic coccus isolated from the blood in my experiments because this organism seems to be more uniform in its various properties and therefore more representative of this group than organisms isolated from other sources. Cocci of the viridans type from the throat may vary considerably in many ways; for example, in pathogenicity, morphology, character of zone on blood agar, etc. It is possible that we have here to deal with a group of organisms whose limits relatively are very wide and I think many strains of cocci of this type isolated from various sources should be tested by anaphylactic reactions with the view of clearing up this point.

I wish to state that Table 1 is not intended to be a complete classification of streptococci. However, I believe a complete and satisfactory classification must rest upon some such conception as is here brought forth. There are undoubtedly organisms of this group other than those mentioned in this paper which may find a place in the series. I have observed cocci, isolated especially from the throat and from sputum, which are different in certain respects from *Streptococcus viridans*, and yet they would appear to occupy a position in the series either just above or just below this organism. I have also observed a very highly hemolytic streptococcus isolated from an alveolar abscess whose growth is very delicate and dry and which I think might be placed in the series just above *Streptococcus hemolyticus*. Furthermore it may very well be that with further study it will be necessary to alter the relative positions of some of the members in the series as it is given above in Table 1.

#### SUMMARY.

The data in this paper are arranged in such a way as to indicate a biologic classification of members of the streptococcus group. Hemolysis, growth on blood agar, capsule formation, solubility in bile, sugar reaction, pathogenic properties in animals and anaphylactic reactions are considered. Transformation of one member into another within certain limits appears to be a not uncommon phenomenon.

## SOME EXPERIMENTS BEARING UPON DROPLET INFECTION IN DIPHTHERIA.\*

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The occurrence of cases of laryngeal diphtheria without involvement of the pharynx is strong evidence in favor of the view that infection sometimes occurs through inhalation of diphtheria bacilli. If infection is by inhalation in these cases, then it seems not unlikely that a certain percentage of the pharyngeal cases are also infected in this manner; for floating particles containing viable diphtheria bacilli may be caught upon the mucous membrane of the pharynx as readily as upon that of the larynx and there is no indication that the latter is more susceptible to infection than the former.

It is conceivable that viable diphtheria bacilli may be inhaled either contained within fine droplets of saliva or attached to particles of dust. This investigation was undertaken with the idea of obtaining a more definite conception than we possess of the part played by the former of these methods in the spread of diphtheria.

That gross particles of saliva and even pieces of membrane are occasionally thrown out by diphtheria patients in coughing is well known, and the danger of infection to one immediately in front of the patient is obvious; but infection in this manner is probably of rare occurrence and is not considered in this paper. The possibility of infection through the inhalation of particles of dust containing diphtheria bacilli also will not be discussed.

The importance of droplet infection in the spread of diphtheria will depend primarily upon the number of droplets containing viable diphtheria bacilli that are emitted by the patients, but not upon this factor alone.

The possible influence of another factor is illustrated in the incidence of epidemics of pneumonic plague. This disease spread with amazing rapidity during the epidemic in Manchuria in the

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winter of 1910-11. On the other hand, though there have been numerous isolated cases of plague pneumonia in India (probably more than 1 per cent of all the plague cases), yet this form of the disease has not assumed epidemic proportions there. It seems probable that droplets containing plague bacilli were thrown out in large numbers in both instances, but that in the hot climate of India the plague bacilli in the droplets quickly suffered death from drying, whereas, in the bitterly cold climate of Manchuria, with its poorly heated dwellings, the bacilli remained alive in the air for a relatively long period of time. This explanation of the occurrence of epidemics of pneumonic plague in cold climates was recently advanced and experimentally supported by Teague and Barber.<sup>1</sup>

The second factor bearing upon the importance of droplet infection in diphtheria is, then, the length of time that the diphtheria bacilli contained in the floating droplets remain alive; the danger of infection is obviously the greater, the longer the living diphtheria bacilli remain suspended in the air in the neighborhood of the patient. This factor will depend primarily upon the resistance of diphtheria bacilli to death from drying and to a less extent upon the condition of the atmosphere with regard to temperature, moisture, and light in the individual case.

I first attempted to determine whether the patients in coughing and talking emit diphtheria bacilli frequently, or only rarely, and whether in large numbers, or only in small numbers. Extensive experiments have been carried out by Flügge, his pupils, and others with reference to the emission of tubercle bacilli, but I have been unable to find any experimental data in the literature with regard to the emission of diphtheria bacilli.

#### MODE OF PROCEDURE.

Loeffler's serum medium was used exclusively. The mixture of ox-serum and glucose broth was solidified in petri dishes about 4 inches in diameter and then sterilized by two further heatings in the Arnold sterilizer. Only freshly prepared plates were used and care was taken that the surface of the medium should not be too dry. Just before the plates were used, the excess of water

<sup>1</sup> *Philippine Jour. Sc.*, 1912 7, p. 157.

was removed with a pipette and then either the plates were kept partially uncovered in the incubator for about half an hour, or circles of sterile blotting paper were applied to the surface of the medium for a few minutes and then removed.

Usually plates were exposed before two or three different cases on each visit to the hospital. The patient was, first of all, requested to expectorate into a sterile empty petri dish and a little of the saliva thus obtained was inoculated upon one of the Loeffler plates by means of a sterile swab. The patient was then requested to talk (to repeat the alphabet, count to 50, and so forth) while an open Loeffler plate was held about three inches from his or her mouth. These two procedures, namely, the culture of the saliva and the exposure of a plate during talking, were always carried out before the patient was asked to cough and before cultures were made from the secretions on the tonsils. The tonsillar cultures were also made upon Loeffler plates in order to obtain a better idea of the number of diphtheria colonies than is afforded by tube cultures. Plates were held in like manner about three inches from the mouth and the patient was told to cough a number of times. If the very young patients did not cough spontaneously, a spatula was sometimes touched against the pharynx to excite coughing; in other instances the child was held lying on its back in bed and the plates were exposed before its mouth while it cried.

In less than an hour and a half after their exposure before the patients, the plates were placed in the incubator. They were examined daily for four or five days, smears from the separate colonies being stained with Loeffler's methylene blue solution. Any colony that contained diphtheria bacilli was recorded as a diphtheria colony, even though there was an abundance of other organisms and only a few diphtheria bacilli present in the stained smear; in other words, mixed colonies containing diphtheria bacilli are included among the diphtheria colonies, in the table. However colonies which apparently contained only diphtheria bacilli were much more frequently met with than these mixed colonies.

For the recognition of *B. diphtheriae* the morphology of the bacillus when stained with Loeffler's methylene blue solution was

alone relied upon. It was not deemed necessary for the purposes of this investigation to carry out fermentation tests or animal inoculations. Therefore a few errors may have crept in, since certain colonies containing diphtheroids may have been recorded in the diphtheria column and, on the other hand, a few colonies containing diphtheria bacilli may have been overlooked; but such errors could not have affected the results materially.

A few of the exposed plates were overgrown with liquefying organisms; such plates were discarded.

In the first 23 cases, cultures of the saliva were not made.

The first plates were exposed on October 9, 1912, and the last on January 4, 1913. The exposures were all made in the well-heated and well-ventilated wards of the Willard Parker Hospital. All of the patients had received diphtheria antitoxin.

Plates were exposed before 54 cases, but those of cases No. 30, No. 39, and No. 40 were not examined. The remaining 51 cases represent 49 different patients, since cases No. 8 and No. 28 were the same patients as No. 5 and No. 24 respectively, plates having been exposed before these two patients both while the tube was in the larynx and after its removal. Of the 49 patients, 29 were males and 20 were females.

In 40 of the 51 cases, cultures of the secretions upon the tonsils were positive and three other cases may be regarded as certainly positive; namely, patient No. 17, who was dying of clinical diphtheria, for which reason the tonsillar culture was not made; patient No. 34, whose tonsillar plate was overgrown by a liquefying organism, but the saliva was found to be positive; and case No. 6, from which diphtheria colonies were found on the exposed plates. Of the eight negative cases, one (No. 28) had been found positive by me four days before, two (No. 38 and No. 50) had been reported positive by the Bureau of Health a few days previous to my examination, and three (No. 19, No. 23, and No. 45) were croup cases. The control cultures of case No. 31 were overgrown with a liquefying organism. The remaining negative case was No. 21. It seems not unlikely, therefore, that diphtheria bacilli were present in small numbers in the throat or larynx of most of these negative cases at the time the plates were exposed before them.

TABLE 1.

CASE NUMBER	AGE IN YEARS	DAYS ILL	MEMBRANE VISIBLE	CONTROL CULTURES		PLATES EXPOSED	RESULTS		REMARKS
				Saliva	Tonsil		Colonies	Diphtheria Colonies	
1	4	6	No	..	+++	3 coughs and crying..... 3 coughs..... Speaking about 20 words.....	10 5 16	1 0 0	
2	8	..	No	..	+	Speaking about 80 words..... 4 coughs..... 4 coughs.....	3 5 12	0 0 1	
3	7(?)	5	No		+++	Speaking about 60 words..... 6 coughs..... 9 coughs.....	8 6 9	0 0 0	
4	3½	4	Yes	..	+++	Speaking 12 words..... Crying..... 4 coughs and crying.....	11 5 13	7 0 0	All of the diphtheria colonies in a single cluster
5	5	8	Yes	..	++	4 attempts at coughing..... 6 attempts at coughing.....	1 6	1 0	Intubated 3 days ago. Tube still in larynx.
6	2½	4	No	..	Negative	Loud crying for about 1 min..... Loud crying for about 1 min..... 7 coughs.....	15 19 19	1 0 1	
7	18	10	No	..	+	Counting 50 and repeating alphabet..... 5 coughs..... 5 coughs.....	32 75 16	0 1 0	
8 (Same patient as No. 5)	5	10	Yes	..	++	8 coughs..... 8 coughs through 4 layers of gauze..... 14 coughs..... 10 coughs through 2 layers of gauze..... 10 coughs..... 10 coughs through 1 layer of gauze.....	19 13 14 45 18 15	0 0 0 0 0 0	Intubated 5 days ago. Ex-tubated a few hours ago.
9	25	6	Yes	..	+++	Counting to 30 and repeating alphabet..... 4 coughs..... 7 coughs..... 9 coughs through 2 layers of gauze.....	12 20 39 14	0 0 0 0	
10	26	3	Yes	..	++	Counting to 50..... 6 coughs..... 10 coughs.....	13 30 18	0 0 0	

TABLE 1—Continued.

CASE NUMBER	AGE IN YEARS	DAYS ILL	MEMBRANE VISIBLE	CONTROL CULTURES		PLATES EXPOSED	RESULTS		REMARKS
				Saliva	Tonsil		Colonies	Diphtheria Colonies	
11	3½	4	..	..	+++	7 attempts at coughing. 11 attempts at coughing.	15 19	1 1	Intubated a few hours ago.
12	2½	6	..	..	++	Attempts at crying. Attempts at crying. Attempts at crying.	7 34 23	1 0 0	Intubated 4 days ago. Coughed up tube today. Voiceless.
13	7	4	Yes	..	+++	One grunt and repeating the alphabet. Counting to 50. Counting to 50.	35 18 18	4 0 1	
14	3	5	..	..	+	5 coughs and talking. 3 coughs. 4 coughs.	22 12 3	0 3 0	Intubated 2 days ago. Tube still in larynx.
15	2½	..	..	..	+	Crying, nostrils compressed. Crying, nostrils compressed. Crying.	3 7 3	0 0 0	Extubated 2 days ago.
16	5	3	Yes	..	+++	Counting to 50. 5 coughs. 8 coughs.	8 7 3	0 0 0	
17	4	3(?)	..	..	..	Breathing about 20 times. Breathing about 20 times.	4 0	0 0	Intubated today. Mortibund.
18	7(?)	7	..	..	+	3 coughs. 4 spontaneous coughs.	6 8	0 0	Extubated this morning.
19	4	9	..	..	Negative	3 natural coughs. 10 coughs. 7 coughs.	About 300 3 7	0 0 0	Extubated this morning.
20	5	6	Yes	..	+++	Counting to 40. 3 coughs. 6 coughs.	5 5 19	0 1 1	
21	6	2	Yes	..	Negative	Crying and 2 coughs, spatula in mouth. Counting to 20. 2 attempts at coughing.	120 10 12	0 0 0	



TABLE 1—Continued.

CASE NUMBER	AGE IN YEARS	DAYS ILL	MEMBRANE VISIBLE	CONTROL CULTURES		PLATES EXPOSED	RESULTS		REMARKS
				Saliva	Tonsil		Colonies	Diphtheria Colonies	
22	7	..	No	..	++	Counting to 50. 5 weak coughs.	63 18	0 0	Carrier for 12 days.
23	1½	5	No	..	Negative	3 coughs 6 coughs 5 coughs 6 coughs 12 coughs	18 8 28 6 8	0 0 0 0 0	Croup. Not intubated.
24	4½	8	..	++	+++	1 attempt at coughing 2 attempts at coughing	9 17	0 0	Intubated yesterday.
25	4(?)	5	..	Negative	+	5 attempts at coughing Talking and attempts at coughing 7 attempts at coughing	11 22 31	1 0 1	Intubated 4 days ago. Tube still in larynx.
26	24	5	Yes	+++	+++	Repeating alphabet and counting 100 by fives twice 10 coughs 7 coughs Talking 3 loud coughs	15 48 12 28 19	0 8 5 1 4	Coughed up cast of trachea just before plates were exposed.
27	4	4	..	..	++	1 cough with spatula in mouth 5 good coughs 3 coughs	11 12 About 150	1 0 0	Intubated today.
28 (Same patient as No. 24)	4½	12	..	Negative	Negative	2 weak coughs 3 natural coughs 5 natural coughs	1 4 0	0 0 0	Intubated 5 days ago. Extubated today. (Control cultures positive 4 days ago.)
29	19	6	Yes	++	+++	Counting to 50 10 loud coughs 5 loud coughs Singing about 30 words 12 loud coughs 8 coughs 7 coughs	1 5 3 2 4 4 15	0 3 0 0 2 4 0	

TABLE 1—Continued.

CASE NUMBER	AGE IN YEARS	DAYS ILL	MEMBRANE VISIBLE	CONTROL CULTURES		PLATES EXPOSED	RESULTS		REMARKS
				Saliva	Tonsil		Colonies	Diphtheria Colonies	
31	7	4	Yes	Negative	Negative	Counting to 50..... Singing about 40 words.....	8 4	0 0	Control cultures overgrown with a liquefying organism.
32	37	7	No	Negative	+	4 strong coughs..... 4 loud coughs..... Counting to 50.....	18 14 22	0 0 0	
33	36	4	Yes	Negative	+++	10 forcible coughs..... 4 coughs..... Counting to 50..... Counting to 50 in a low voice.....	25 11 5 10	1 0 0 0	
34	18	4	Yes	++	Plate liquefied	7 good coughs..... Counting to 50.....	30 15	1 0	
35	8	3	Yes	+++	+++	Talking..... 6 weak coughs..... 5 weak coughs.....	10 20 0	0 0 0	
36	4½	..	Yes	++	+++	Coughing twice and crying..... Crying..... Crying..... Talking.....	50 0 0 7	2 0 0 0	
37	34	5	Yes	+++	+++	6 loud coughs..... Counting to 50 slowly, distinctly..... Speaking about 20 words..... Speaking about 30 words..... 4 coughs..... 5 coughs.....	20 23 20 2 0 0	0 1 1 0 0 0	
38	28	4	No	Negative	Negative	9 loud coughs..... 3 loud coughs.....	60 0	0 0	
41	5	5	..	+	++	Whispering and counting to 50..... 8 half-spontaneous coughs..... 8 natural coughs..... 12 coughs.....	10 4 6 5	1 0 0 0	Intubated 2 days ago. Tube still in larynx.

TABLE I—Continued.

CASE NUMBER	AGE IN YEARS	DAYS ILL	MEMBRANE VISIBLE	CONTROL CULTURES		PLATES EXPOSED	RESULTS		REMARKS
				Saliva	Tonsil		Colonies	Diphtheria Colonies	
42	9	5	Yes	++	++	Counting to 100 and repeating alphabet 6 attempts at coughing. . . . . 12 attempts at coughing (a small piece of membrane thrown upon plate). . . . . 15 attempts at coughing. . . . .	15 24 53 325	0 0 23 47	
43	16	4	Yes	++	+++	Counting to 50, repeating alphabet and Lord's prayer. . . . . 5 attempts at coughing. . . . . 6 attempts at coughing. . . . . 10 coughs. . . . .	10 4 8 42	0 1 1 4	
44	30	3	Yes	+	++	Counting to 50 and talking . . . . . Singing about 50 words. . . . . 8 weak attempts at coughing. . . . . 12 attempts at coughing. . . . .	16 15 6 24	0 0 0 1	
45	5	2(?)	No	Negative	Negative	Hoarse crying. . . . . Hoarse crying. . . . . 5 coughs. . . . . 10 coughs. . . . .	3 8 24 13	0 0 0 0	Croup. Not intubated.
46	4½	5	..	+	++	Hoarse crying. . . . . Hoarse crying. . . . . Hoarse crying. . . . . Hoarse crying. . . . .	42 18 36 20	15 0 1 0	Croup. Not intubated.
47	2½	4	No	++	++	Heavy breathing. . . . . 3 weak coughs, spatula in mouth. . . . . 4 weak coughs, spatula in mouth. . . . . 2 weak coughs, spatula in mouth. . . . .	22 1 2 2	0 0 0 0	Intubated 2 days ago. Tube still in larynx.
48	6	7	?	+	++	Counting to 30 and talking. . . . . 2 weak attempts at coughing. . . . . 7 coughs after drinking water. . . . . 8 attempts at coughing. . . . .	11 16 28 4	2 5 6 0	Intubated this morning.

TABLE 1—Continued.

CASE NUMBER	AGE IN YEARS	DAYS ILL	MEMBRANE VISIBLE	CONTROL CULTURES		PLATES EXPOSED	RESULTS		REMARKS
				Saliva	Tonsil		Colonies	Diphtheria Colonies	
49	8	5	Yes	++	+++	Counting to 50 and repeating alphabet... 13 attempts at coughing... 9 good coughs... 10 good coughs... 7 good coughs... 5 good coughs...	4 2 13 12 6 2	1 1 1 1 1 0	
50	24	5	..	Negative	Negative	Counting to 50 and repeating alphabet... 5 forcible coughs... 5 forcible coughs... 7 forcible coughs... 9 coughs...	4 34 28 80 75	0 0 0 0 0	Culture reported positive 2 days ago.
51	2	4	Yes	++	+++	Loud crying for 3 min... Loud crying for 2 min... Loud crying for 1 min... Loud crying for 2 min...	3 2 1 1	0 0 0 0	
52	27	4	Yes	Negative	+	Counting to 30 and talking... 12 attempts at coughing... 6 weak attempts at coughing... 6 attempts at coughing... 18 weak attempts at coughing...	4 29 7 26 2	0 0 0 0 0	
53	19	5	Yes	++	++	Counting to 50 and alphabet... 6 coughs... 10 coughs... 4 coughs...	1 14 24 9	0 0 1 0	
54	11	3	Yes	++	+++	Counting to 50, repeating alphabet and talking... 12 coughs, some natural... 6 strong attempts at coughing... 10 coughs... 12 coughs...	2 8 0 8 15	0 0 0 4 6	

+++ = confluent growth of diphtheria bacilli.

++ = numerous scattered diphtheria colonies.

+ = a few diphtheria colonies.

To summarize the results given in the preceding table: of the 51 cases, 28 were proved to have thrown droplets containing viable diphtheria bacilli upon at least one of the plates exposed before them. Of 180 plates exposed before the patients, 48 were positive for diphtheria bacilli. In other words, more than half of the cases emitted diphtheria bacilli in coughing or talking and more than one-fourth of the plates exposed showed diphtheria colonies. If the eight cases, in which the control cultures were negative, are omitted, then 65 per cent of the cases emitted diphtheria bacilli and 31 per cent of the exposed plates were positive. When it is borne in mind that the plates were exposed before the patients only for very short periods (from a few seconds to two or three minutes), that many of the attempts at coughing were less forcible than spontaneous coughs, and finally that only a fraction of the emitted droplets were caught upon the plates, these results are very striking. For the sake of clearness the results are summarized in tabular form.

TABLE 2.

	All Cases	All Cases Except Those with Negative Control Cultures*	Cases in Which Control Cultures Contained Numerous Diphtheria Colonies†
Total number of cases.....	51	43	34
Number of cases that emitted diphtheria bacilli.....	28	28	23
Number of cases that did not emit diphtheria bacilli.....	23	15	11
Percentage of cases that emitted diphtheria bacilli.....	55	65	68
Total number of plates exposed.....	180	153	124
Number containing diphtheria colonies....	48	48	41
Number containing no diphtheria colonies..	132	105	83
Percentage of positive plates.....	27	31	33
Average number of diphtheria colonies on positive plates.....	3.75	3.75	4.2

\* Cases Nos. 10, 21, 23, 28, 31, 38, 45, and 50 omitted.

† Cases Nos. 2, 6, 7, 14, 15, 17, 25, 32, and 52 omitted in addition to the eight cases with negative cultures.

Thirty of the 48 positive plates contained only a single diphtheria colony each. Only three of the plates showed more than eight diphtheria colonies, and two of these were exposed under rather artificial conditions (the patient grunting and blowing instead of coughing) and may, therefore, be disregarded.

Four of the cases will be described in somewhat more detail than in the table.

*Case 26.*—A man, 24 years old, had been ill 5 days. Diphtheritic membranes on the uvula and both tonsils. Just before my arrival at the hospital, the patient had coughed up a tubular cast of the trachea about three and a half inches long. Cultures from the tonsil and from the blood-stained sputum showed a confluent growth of diphtheria. The patient sat up in bed with assistance and talked in a fairly loud voice. The coughs were loud and strong. At each cough I could feel the expired air striking forcibly against the exposed plate. Diphtheria colonies developed upon four of the five plates exposed, but the largest number of diphtheria colonies upon a plate was only eight.

*Case 29.*—A man, 19 years old, had been ill 6 days. An extensive diphtheritic membrane on the posterior wall of the pharynx and uvula. The patient was strong enough to sit up in bed without assistance and talked in a loud conversational tone. Good coughing, a strong current of air striking the plate with each cough. The culture from the throat showed a confluent growth of diphtheria, that of the saliva about 150 scattered diphtheria colonies upon the plate. Seven plates were exposed, two of which showed two and three diphtheria colonies respectively.

*Case 42.*—A boy, 9 years old, had been ill 5 days. He had apparently had an extensive membrane, though I did not determine to what extent the membrane was still present. Throat badly swollen. The patient sat up in bed and made earnest and forcible attempts at coughing but did not actually cough. His attempts consisted largely in grunting and blowing. The conditions were, therefore, decidedly artificial and no great importance can be attached to the results. Large numbers of diphtheria colonies developed upon two of the four plates exposed.

A small piece of membrane was thrown upon one of the plates. This was the only instance in the course of these experiments in which a gross particle was seen to strike the plate.

*Case 51.*—A two-year-old male baby that had been ill 4 days. An extensive diphtheritic membrane covered the posterior pharynx and the tonsils. The control culture from the tonsils showed a confluent growth of diphtheria. A little saliva swabbed from beneath the tip of the patient's tongue brought about the development of about 75 scattered diphtheria colonies on a Loeffler plate. The baby was held lying on its back in bed by the nurse and plates were exposed before its mouth, for 1 minute, 2 minutes, and 3 minutes respectively, while it cried in a loud voice. No diphtheria colonies and very few other colonies developed on the plates.

These experiments indicate that droplets containing viable diphtheria bacilli are emitted very frequently by diphtheria patients in talking and coughing, but usually in small numbers only.

Since coughing consists in subjecting the air behind the closed vocal cords to high pressure and then suddenly opening the cords, it was thought that perhaps laryngeal cases would emit vastly more droplets containing diphtheria bacilli than pharyngeal or tonsillar cases. If this were true, obviously the laryngeal cases

should receive special attention with regard to isolation, nursing, etc. It was found, however, that the laryngeal cases investigated did not emit appreciably more droplets containing *B. diphtheriae* than did the other cases. But since many of the laryngeal cases had been intubated and hence could not cough in the manner indicated above, it was decided to attempt to throw further light upon this question by carrying out experiments with *B. prodigiosus*.

It was planned to determine whether more droplets containing *B. prodigiosus* were emitted on coughing and talking after swabbing the larynx with a suspension of this bacillus than after swabbing the pharynx and tonsils or the mouth with the same suspension. Dr. Wells P. Eagleton kindly agreed to swab my larynx. Agar plates, upon which *B. prodigiosus* produced red colonies, were exposed about two or three inches from the mouth during coughing and talking. "Now is the time for all good men to come to the aid of their party," a sentence of 16 words, containing most of the letters of the alphabet, was selected for the short exposures during talking, and the Lord's Prayer, the alphabet, and counting to 50 (about 150 words) for the longer ones.

TABLE 3.

	AFTER SWABBING LARYNX	AFTER SWABBING UVULA, TONSILS, AND PILLARS	AFTER SWABBING TEETH, LIPS, TONGUE, AND PALATE
	Prodigious Colonies	Prodigious Colonies	Prodigious Colonies
16 words.....	0	0	0
2 coughs.....	13	3	11
150 words.....	0	0	5
5 coughs.....	4	2	32
Control.....	0	0	0
16 words.....	0	0	1
2 coughs.....	0	1	11
150 words.....	0	0	2
5 coughs.....	0	1	4
Control.....	0	0	0

The greater part of one agar slant of *B. prodigiosus* was suspended in about 10 c.c. of 0.8 per cent sodium chlorid solution. The larynx was swabbed with this suspension, care being taken not to contaminate the mouth or pharynx during the procedure. Plates were then exposed during coughing and talking. Control plates were held before the mouth during quiet breathing. After

that the uvula, tonsils, and pillars were swabbed with the same suspension and plates were exposed in the same manner as before. Finally the teeth, lips, tongue, and palate were swabbed with the suspension of *B. prodigiosus* and plates were exposed in like manner for the third time. The result of this experiment is given in Table 3.

It is seen from Table 3 that no colonies of *B. prodigiosus* developed on the plates exposed during talking and only a few colonies on those exposed during coughing after the larynx had been swabbed. In this experiment much larger amounts of the suspension were used in swabbing the pharynx and mouth than in swabbing the larynx. For this reason further similar experiments were performed, approximately the same amount of the suspension having been used at each swabbing. Dr. J. L. Dias kindly applied the prodigiosus suspension and Dr. J. J. Smith and Dr. E. W. Sprague acted as subjects.

TABLE 4.

	DR. SPRAGUE AS SUBJECT	DR. SMITH AS SUBJECT		DR. SPRAGUE AS SUBJECT	DR. SMITH AS SUBJECT
	Prodigious Colonies	Prodigious Colonies		Prodigious Colonies	Prodigious Colonies
LARYNX SWABBED:			TONSILS, UVULA, AND PILLARS		
16 words.....	0	4	RESWABBED:		
150 words.....	0	15	2 coughs.....	2	7
16 words.....	0	1	5 coughs.....	48	25
150 words.....	0	7	2 coughs.....	275	3
			5 coughs.....	117	6
LARYNX RESWABBED:			TONGUE, LIPS, AND MUCOSA OF		
2 coughs.....	6	53	CHEEKS SWABBED:		
5 coughs.....	34	14	16 words.....	3	2
2 coughs.....	1	2	150 words.....	11	19
5 coughs.....	2	2	16 words.....	5	2
			150 words.....	20	23
TONSILS, UVULA, AND PILLARS SWABBED:			TONGUE, LIPS, AND MUCOSA OF CHEEKS RE- SWABBED:		
16 words.....	0	0	2 coughs.....	10	2
150 words.....	2	3	5 coughs.....	8	10
16 words.....	0	0	2 coughs.....	7	13
150 words.....	0	2	5 coughs.....	1	12

The results shown in Table 4 agree with those of the first experiment. These experiments all indicate that in talking and coughing no more droplets are thrown out from the larynx than from the



mouth and that if a larger series of suitable cases of laryngeal diphtheria had been investigated, the results would not have been materially different from those obtained.

It was shown by Hutchison<sup>1</sup> that *B. prodigiosus* contained in fine droplets may be carried 53 meters along a corridor and up two flights of stairs; in another experiment he found that it was carried by a breeze over as great a distance as 600 meters. Other investigators have obtained similar results.

Since *B. diphtheriae* is much more resistant to death from drying than *B. prodigiosus*, we may assume that, under similar circumstances, it would be carried over even greater distances.

Many investigators have shown that *B. diphtheriae* is very resistant to drying under various conditions, but there are few experiments indicating the relative resistance of *B. diphtheriae* and *B. prodigiosus*. Kirstein<sup>2</sup> sprayed suspensions of different bacteria and allowed only the finer droplets to settle upon empty sterile petri dishes, which were then kept at room temperature. Culture medium was added to the plates at various intervals of time and the number of developing colonies recorded. He sums up his results as follows:

Kind of Bacteria	Average Time of Death of Bacteria after Spraying
<i>B. prodigiosus</i> . . . . .	24 hrs.
<i>B. typhosus</i> . . . . .	24 hrs.
<i>B. diphtheriae</i> . . . . .	24-48 hrs.
<i>Staphylococcus aureus</i> . . . . .	8-10 days

The following experiment, which I performed, demonstrates more strikingly the greater resistance of *B. diphtheriae*. Suspensions of the different bacteria to be tested were prepared in 0.8 per cent sodium chlorid solution and made of approximately the same degree of cloudiness as judged by the eye. Bits of sterilized absorbent cotton were dipped into the suspensions, thoroughly squeezed out between the thumb and finger, and quickly rubbed over the surface of about a dozen carefully cleaned and sterilized slides. The slides lying upon a piece of blotting paper, the surface of which had been sterilized in the free flame, were then placed in an incubator at 37° C. At intervals slides were removed and placed face down upon agar plates; after an hour they were moved back and forth across the surface of the agar and then removed. The plates were incubated for several days and the developing colonies counted with the results shown on p. 413.

The vast number of experiments in the literature upon the resistance of bacteria to drying affords a rather confused picture, because they were performed under such widely different conditions. It would seem to be highly desirable to classify the bacteria into groups with reference to their resistance to drying by the use of some such method as that just described.

<sup>1</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1901, 36, p. 223.

<sup>2</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1902, 30, p. 163.

Interval	Cholera	Prodigiousus	Typhoid	Diphtheria	Staphylococcus
At once (control)....	Colonies innumerable	Colonies innumerable	Colonies innumerable	Colonies innumerable	Colonies innumerable
2 min.....	0	..	..	..	..
1 hr.....	..	140	..	..	..
1 1/2 hrs.....	..	60	..	..	..
2 hrs.....	..	..	Numerous	Numerous	Numerous
3 hrs.....	..	2	..	..	..
4 hrs.....	..	6	..	..	..
9 1/2 hrs.....	..	2	125	64	..
20 hrs.....	..	1	0	28	..
28 hrs.....	..	0	14	25	..
36 hrs.....	..	..	6	40	600
48 hrs.....	..	0	1	4	406
60 hrs.....	..	..	0	3	..
3 days.....	..	..	0	0	130
4 days.....	..	..	1	1	04
5 days.....	..	..	1	0	102
6 days.....	..	..	..	..	147
7 days.....	..	..	..	..	41

Working in the tropics, Dr. Barber and the writer found that, after being sprayed, *B. prodigiousus* disappeared from the air more rapidly than previous experiments carried out in temperate climates would lead one to expect. By spraying in an atmosphere saturated with water vapor, we showed that this disappearance of living prodigiousus bacilli from the air was due to death from drying and not to settling. We also sprayed *B. prodigiousus* in a cold storage room at a temperature of 12° C., and were the first to point out the marked effect of the low temperature in prolonging the life of the bacilli in suspended droplets. This last observation has, without doubt, an important bearing upon droplet infection in general. An individual with influenza must be far more dangerous in a cold room than in a warm one; for we know that the influenza bacillus is readily killed by drying and the longer the bacilli remain alive in the air, obviously the greater the danger of infection. It seems not unlikely that the increased danger of infection is in part responsible for what is often spoken of as the "lowered resistance" of the individual from remaining in an insufficiently heated room. Also, one meets with the argument that droplet infection plays no part in certain diseases, because medical students and others visit hospitals containing patients with these diseases and escape infection. This argument is fallacious for the reason that a well-heated and well-ventilated hospital ward offers different conditions with regard to the persistence of droplets in the air from what

would be met with, for example, in a poorly heated tenement house in a cold climate.

#### CONCLUSIONS.

In talking and coughing, diphtheria patients frequently emit droplets containing viable diphtheria bacilli, but they emit such droplets usually in small numbers only.

Patients with laryngeal diphtheria apparently do not throw out many more droplets containing diphtheria bacilli than do pharyngeal cases. In agreement with this observation, it was found that after swabbing the larynx with a suspension of *B. prodigiosus*, no more droplets containing this bacillus are thrown out in coughing and talking than after swabbing the pharynx and mouth.

The diphtheria bacillus is much more resistant to death from drying than *B. prodigiosus*. Hence, the former would tend to remain alive in suspended droplets longer than does *B. prodigiosus* in the numerous series of experiments in which it has been used.

It is pointed out that bacteria in suspended droplets would remain alive much longer in poorly heated tenements during cold weather than is indicated by laboratory experiments performed at higher temperatures.

It is believed that the data furnished by these experiments will be of aid to the epidemiologist and to the sanitarian in arriving at a correct estimate of the importance of the part played by droplet infection, as compared to other modes of infection, in causing the spread of diphtheria.

I am greatly indebted to Dr. William H. Park and Dr. M. A. Wilson for placing the material of the Willard Parker Hospital at my disposal. I wish also to thank Drs. Eagleton, Dias, Sprague, and Smith, of Newark, N.J., for their assistance in carrying out the experiments with *B. prodigiosus*.

## EPIDEMIOLOGIC DIAGNOSIS AND MANAGEMENT OF TYPHOID FEVER.\*

C. J. HUNT.

*(From the Pennsylvania State Department of Health, Harrisburg, Pennsylvania.)*

The purpose of this contribution is to present particularly the results of application of all available measures in an epidemic of typhoid fever; however, the diagnosis of the source and the associated studies which were made in this instance are of sufficient importance to warrant their presentation.

In accordance with the instructions of Dr. Samuel G. Dixon, Commissioner of Health, the writer proceeded to Troy, Bradford County, Pennsylvania, on October 12, 1912, in order to investigate the reported prevalence of the disease in question.

The Borough is a very old one and the citizens are, for the most part, retired agriculturists, dairymen or employees of three principal industries, the Troy Engine and Machine Company, the Troy Tanning Company, and the Troy Creamery Company. From a financial point, the Borough is considered the most thriving municipality in Bradford County. There are many wealthy residents. The population has varied but little during a period of 30 years; in 1890 the United States Census Reports recorded a population of 1,307; in 1900, 1,450; and in 1910, 1,288. The census made by inspectors of the State Department of Health during October, 1912, recorded a population of 1,343.

This Borough has previously been practically free from typhoid fever; one case was reported during 1911 and none during the months of 1912 prior to the epidemic. The township of Troy surrounds the Borough and the major portion of the watershed of the West Branch of Sugar Creek. During a period of 10 years there have been but two cases of typhoid fever reported from premises located on the West Branch of Sugar Creek and its tributaries. The first case occurred during 1904, and the patient has not resided on the watershed during the past three or four years. The second

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case was reported on March 1, 1912, and will be referred to later in a consideration of the source of the epidemic. In addition, four cases were reported during February and one each during the months of May and June, 1912, but these cases have been definitely shown to bear no relation to the epidemic.

Up to and including October 12, but 24 cases of typhoid fever had been reported in the Borough by the local physicians. A conference with the members of the Board of Health and the physicians practicing in the Borough was held immediately after arrival; a list of those who were suspected of having the disease was made up in detail so that a census could be made at once. The form used in securing the detailed information relative to these cases is one which has been in use by the Department of Health during a period of two years and has been found entirely satisfactory.

Form 66. COMMONWEALTH OF PENNSYLVANIA, DEPARTMENT OF HEALTH. No.....

CENSUS CARD

Patient.....Township.....County.....  
 Householder.....Address.....  
 Owner.....Address.....  
 Age.....{ Male Black Married Widowed } Occupation .....  
                   { Female White Single }  
 Name and address of employer or school.....  
 Where taken sick?.....Date of first feeling sick.....  
 Doctor's name and address.....Date of first visit.....  
 Nurse's name.....How long attending.....  
 Total population of household.....No. of other cases in household.....  
 Visitors to premises within 30 days? Yes. No. Name.....Address.....  
 Where have visits been made within 30 days?.....  
 Visits to other cases? Yes. No. Food or drink taken? Yes. No.....  
 Sick room screened? Yes. No. Water used at home.....at work.....  
 Other water used within 30 days.....  
 Milk supplied by.....In cans. Bottles. How long from can?.....  
 Other sources within 30 days.....  
 Use of milk { Beverage, Yes. No.  
                   { Dressing, Yes. No.  
                   { Coffee or tea, Yes. No.  
 To whom is milk furnished?.....  
 Ice cream within 30 days? Yes. No. Where purchased?.....  
 Shell fish within 30 days? Yes. No. Where purchased?.....

\* When food or drink has been taken during visit give data under Remarks.

Uncooked vegetables or fruits within 30 days? Yes. No. Where grown?.....  
 Name of dealer supplying ice.....Where harvested?.....  
 Drinking cups, table ware, etc. boiled? Yes. No. Sewer connection. Yes. No.  
 Privy? Yes. No.  
 How are stools and urine disinfected?.....  
 Disposal of stools and urine. Sewer. Privy. Cesspool. Buried.....  
 Are stools and urine at any time exposed to insects, flies, etc.? Yes. No.  
 Garbage disposal.....Kitchen waste.....Wash water.....  
 Have they unslaked or chlorinated lime? Yes. No. What other disinfectants?..  
 .....  
 How and where used?.....  
 Animals—Cats, Dogs, Goats, Birds, Chickens, Rats, Mice?.....  
 Circular left.....House placarded.....  
 Abatement notices.....What for?.....  
 Remarks:

Data obtained by:

The census is made by trained inspectors, who report daily to the officer in charge and dictate the details of each case to an inspection clerk. From the latter's records the tabulated results are compiled.

#### EPIDEMIOLOGY.

The results as tabulated at the end of the epidemic conform to the findings following the census of 41 cases made on October 12 and 13 by five special inspectors and one sanitary inspector. The compiled tables contributed certain interesting facts to the diagnosis of an epidemic; for the sake of brevity, only the general results are presented.

There were 229 cases of typhoid fever which were infected in the Borough of Troy, 225 of which were primary and 4 secondary cases. The careful census of the town gave a total tabulation of 1,343 persons residing in 367 premises. Thirty-two cases were located in other health jurisdictions. Of these, 122 or 33.24 per cent housed persons ill with the disease, the distribution being as follows:

72 houses with 1 case
32 " " 2 cases
9 " " 3 "
3 " " 4 "
2 " " 5 "
1 house " 6 "

The tabulation of the age periods is as follows:

Years of Age	Cases	Percentage
0-5.....	13	5.6
5-10.....	36	15.28
10-30.....	90	43.24
30-40.....	34	14.84
40-50.....	23	10.04
50-60.....	15	6.55
60-70.....	9	3.93

The study of this table brings out certain interesting factors in relation to susceptibility. The large percentage of cases between the 10th and 30th years indicates that the source of transmission was water-borne, but the total range of susceptibility suggests some other factor, common to all, which is not usually found in water-borne epidemics. In practically all water-borne epidemics in Pennsylvania, from 60 per cent to 70 per cent and occasionally over 80 per cent of the cases have been between 10 or 15 and 30 years of age. In milk- or ice-cream-borne epidemics, the predominant age is well below 15 years. As will be shown, these two possible agents were excluded. The disease was fairly evenly distributed between males and females, 127 of the former and 102 of the latter being reported. Occupation was not a factor, as every type of employment was represented among those sick. The most probable factor is that the disease has not been endemic in that region, and the residents have not had that "accustomance" to repeated minimal doses of the toxins of the specific microorganisms.

This theory has some confirmation in the total morbidity which amounted to 17 per cent. In no other reported epidemic has there occurred so high a relative morbidity. The epidemic at Plymouth, Luzerne County, Pennsylvania, during 1895, showed a morbidity of about 11 per cent; that in Butler, Butler County, Pennsylvania, during 1903-4 was about 7 per cent of the total population. The degree of pollution of the water-supply would not account for the high morbidity; if it were possible to draw "a curve of pollution," the latter would not, though marked, present the excessive pollution curve shown in the Coatesville epidemic during February, 1912, in which the morbidity was 2 per cent of those exposed. Hence, we believe that these factors are illustrations of the absence of an acquired tolerance or immunity.

The 229 cases were supplied with milk from 66 different and 1 undetermined sources. Thirty-nine were supplied by their own cows and 189 by various dealers. There were 5 principal milk routes, only one of which proved important enough to mention. This man supplied 157 families, in which there occurred 87 cases of typhoid fever. His wife had suffered with the disease about 20 years previously, and prior to the epidemic period had washed the bottles and other utensils. Careful and repeated examination of the feces and urine failed to recover any pathogenic member of the typho-colon group. At some time within the incubation period, 108 cases had used ice cream. In 15 cases it was "home made" from milk and cream obtained from cows maintained on the premises. In 93 cases it was obtained from 10 local dealers; 55 ate ice cream from 3 principal dealers who purchased their supply from a manufacturer in Elmira. An investigation of the latter's plant proved negative.

Twenty-five, or 10.91 per cent, used shell fish, purchased at 6 different stores or restaurants; 3 of them obtained a supply from the same dealer, but the small number made it unnecessary to follow out the bacteriological examinations.

Ice was readily excluded from consideration as it was used by but few families. The supply came through one dealer from the West Branch of Sugar Creek below the municipal water collecting system and had been harvested during January and February, 1912. Bacteriological analysis of this ice gave negative results for *B. coli* and the highest total count was 17 per 1 c.c. However, specimens of water collected from the pond during December, 1912, gave the following counts:

	Total per c.c.	<i>B. coli</i> per c.c.
North End.....	2,500	320
South End.....	2,000	400

Of the patients, 226 had used the municipal water; 3 had not used that water, but were nurses who were secondary cases; 168, or 74 per cent, used no other water; 51, or 22.5 per cent, used well or spring supplies in addition to the Borough supply, but studies of physical environment and bacteriological analyses excluded them as transmissive agents; 10 obtained water from neighboring



municipal supplies to which no cases of the disease have been traced.

An analysis of the conditions relative to the supply shows that it was designed to be obtained from a system of springs (Fig. 1), the water of which was piped to a common point known as the upper collecting reservoir and to which point was also collected the ground waters from various points in a natural gravel formation, all being



FIG. 2.—The upper collection reservoir, 35 feet distant from creek bank. Piping of drilled well noted in background.

in a vale through which flowed the West Branch of Sugar Creek. These sources have been purchased by the Borough, and the three acres of land were known as the "Spring Lot." This three-acre tract is located one and one-half miles west of the Borough, and the water is delivered by gravity to two distributing reservoirs situated at the west edge of the Borough; at this point an auxiliary supply was obtained from a drilled well. A small low-surface district in the south end of the town furnished a small supply from the spring owned by the Pennsylvania Railroad Company about a mile

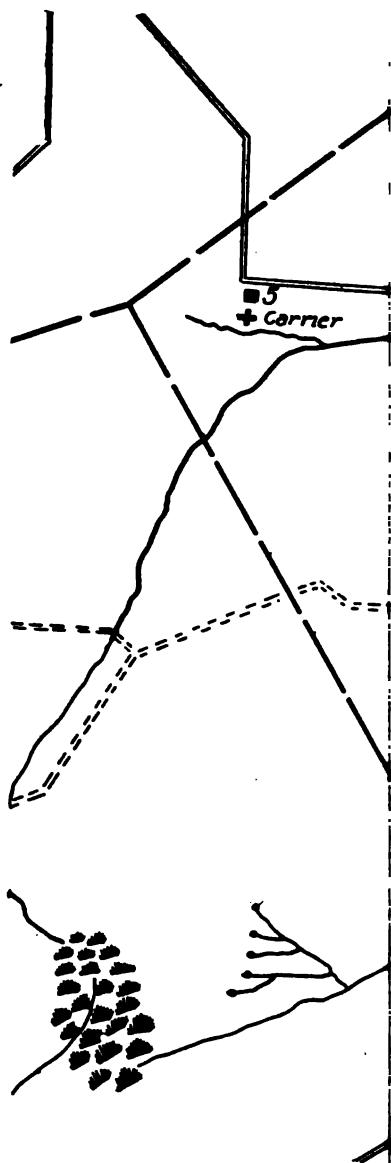


FIG. 1.—Troy Borough and Troy Borough  
on the banks of Spring Run. Case 5 in the same



southeast of the Borough and delivered to and connected with the first system by gravity.

At the Spring Lot there were six shallow springs and two 8-inch diameter, flowing, drilled wells, 100 and 194 feet deep respectively. The water was collected in a system of open-joint tile pipes and conveyed to a common point called the upper reservoir (Fig. 2), from which it passed by gravity through an 8-inch main to a distributing



FIG. 3.—West Branch of Sugar Creek. Uranine, placed in a pocket near the large stones in the foreground, appeared in the reservoir within 33 seconds. The perforated barrel is located in the creek bed to the extreme left.

reservoir at the edge of the Borough. In addition, there were two creek intakes at the Spring Lot: the one a perforated barrel sunk into the bed of the stream (Fig. 3), covered with flag stone, while the other was an underground concrete diverting wall leading under the bed of the stream from a small collecting chamber located in a meadow on the opposite side of the creek. The intended purpose of this wall was to intercept the ground waters and to deliver them to the upper collecting reservoir. Whether intentionally or otherwise, direct communication was made with the creek.

The sodium salt of fluorescin (uranine) placed in the creek at this point appeared in the upper reservoir (35 feet distant) within a period of 33 seconds, while the same dye, when placed in the perforated barrel, appeared in the lower collecting reservoir (550 feet distant) within 20 minutes.

The normal flow from the Spring Lot, with the creek waters shut out, amounted to approximately 80,000 gallons in 24 hours. This

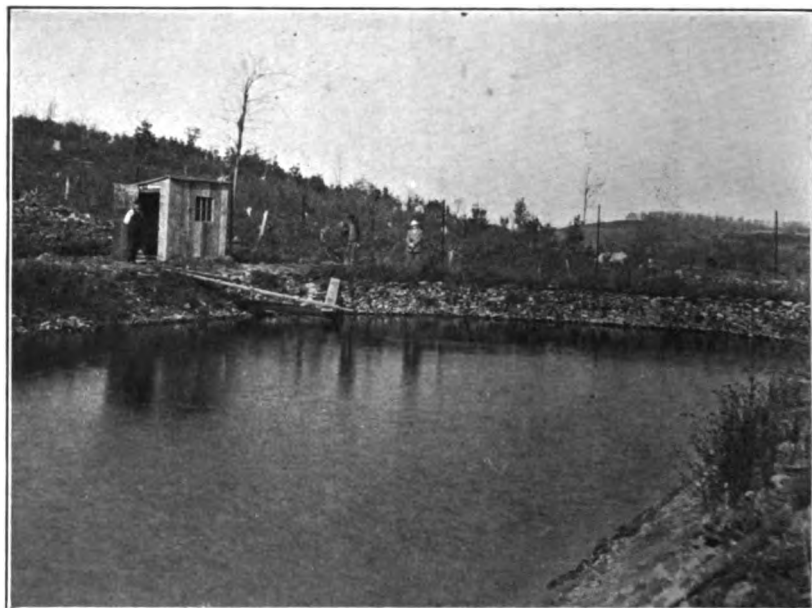


FIG. 4.—Open distributing reservoir and discharge pipe from collection system in the foreground. The pump station for drilled well at this point and the covered reservoir are shown in the background.

was markedly increased by rises, thus indicating the surface nature of the supply. The drilled well at the distributing reservoirs was 4 inches in diameter and 265 feet deep; it was used as an auxiliary supply, pumped by air and had a capacity of about 50,000 gallons during the daily pumpage. The two distributing reservoirs (Figs. 4 and 5), located at the west edge of the Borough had a total storage capacity of 200,000 gallons. All of the water, except from the Pennsylvania Railroad supply, passes through these reservoirs.

Extensive repairs and alterations had been made, the work

beginning late in June and ending on or about September 10. During this period 14 men were employed more or less constantly, and the hygiene at this time was open to suspicion. However, none had previously suffered an attack of typhoid fever, and the history of the other abdominal disturbances was considered too vague to be reliable.

During the winter of 1911-12, there were 4 cases of typhoid

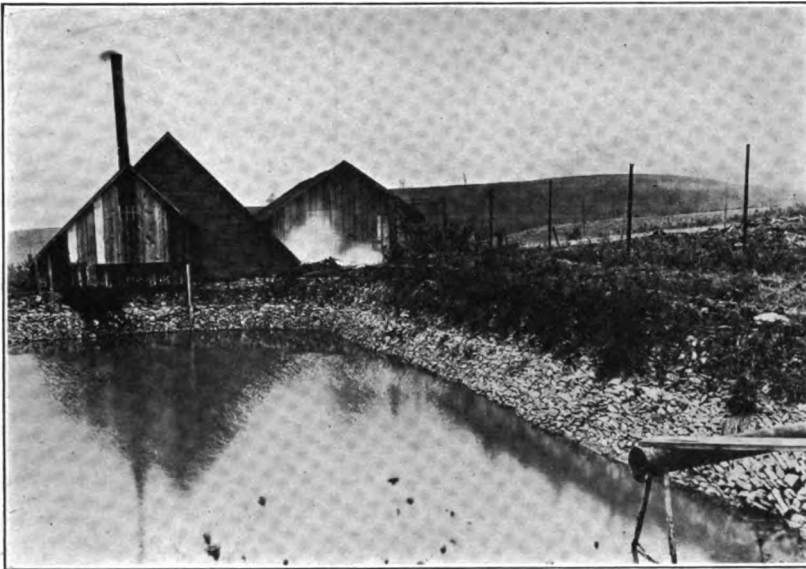


FIG. 5.—The open distributing reservoir, the discharge pipe from the collecting system, and the temporary building erected to house a temporary hypochlorite of lime treatment plant. All water was treated at this point.

fever in one family who lived on the banks of the West Branch of Sugar Creek about 1 mile below the municipal source of water-supply. One W.S., employed on that farm, was taken ill late in the month of February, 1912. His onset is officially recorded as March 4, on which date he returned to the home of his father who lived on a small tributary to the West Branch of Sugar Creek, known as Spring Run. This patient was placed under quarantine immediately upon arrival; a trench was dug for the disposal of the excreta, all of which were thoroughly disinfected with fresh unslaked lime prior to burial in the trench. The trench was located adjacent

to the privy vault, which was about 100 feet distant from and about 50 feet in elevation above Spring Run. There is no evidence that storm water, except under extraordinary conditions, would cause drainage from these points into the stream. However, W.S. worked in harvest fields through which Spring Run and a portion of the West Branch of Sugar Creek flow; during that period and subsequently, he states that at times discharges were deposited in the fields adjacent to the stream. As this was the only authentic case of typhoid fever occurring on the watershed, specimens of feces and urine were collected on October 15, 1912, which, after careful examination, proved to be negative. On November 12 additional specimens were procured, and from the feces, *B. typhosus* was identified by all the required iso-murtoric tests. The type recovered was subsequently used (because of its agglutinability) to study the sera of certain cases from the same epidemic.

In addition to the pollution from this source, there were 7 privies, 1 cesspool, and 27 miscellaneous sources of pollution, including pigpens, barnyards, and kitchen wastes. There was a population of 35 in an area of 3 square miles on the watershed above the point of collection of the Borough water-supply.

The history of weather conditions, according to unreported observations, indicates that there was an unusual precipitation during the summer, particularly in association with severe electric storms. The West Branch of Sugar Creek is subject to quick rises and rapid falls, as is usual in deforested regions. It was not possible to secure observations made in or near the Borough, and the records from the United States Weather Bureau Service, taken at Wellsboro, Tioga County, which is 28 miles west; at Elmira, N.Y., which is 25 miles north; at Towanda, Bradford County, which is 22 miles east, and at Williamsport, Lycoming County, which is 50 miles south, show a rather remarkable correlation in the dates and amounts of precipitation. The observations which bore a direct relation to the incidence of the epidemic are as follows:

	September 15-16	September 22-25
Wellsboro.....	1.59 in.	1.06 in.
Elmira.....	1.05 in.	2.38 in.
Towanda.....	0.45 in. (4 hrs.)	2.55 in.
Williamsport.....	0.24 in.	2.38 in.





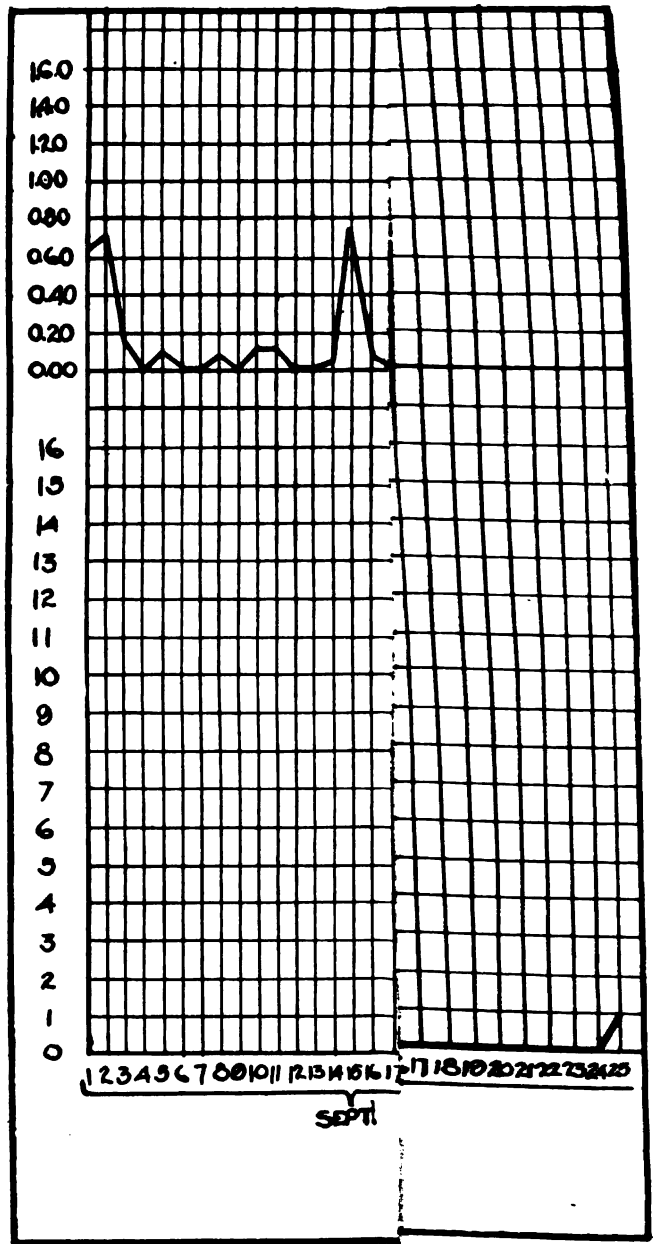


FIG. 6.—The upper curve shows the <sup>daily</sup> mortality.

Representative analysis of specimens obtained from various portions of the system are noted in the following table:

	Total Bacteria per c.c.	<i>B. coli</i> per c.c.
Raw water, W. Branch Sugar Creek .....	240	2
Inlet Pipe, Upper Collecting Basin, Spring Lot .....	10,000	1
Inlet Pipe, Lower Collecting Basin, Spring Lot .....	200	1
Lower Collecting Basin .....	100	1
Inlet, Lower Collecting Basins .....	720	2
Open Reservoir (Distributing) .....	60	2
Covered Reservoir (Distributing) .....	9,500	14
Up-Stream Drilled Well, Spring Lot .....	300	0
Down-Stream, " .....	200	0
Spring No. 1, Spring Lot .....	40	0
Spring No. 2, Spring Lot .....	100	0
Spring No. 3, Spring Lot .....	35	0
Spring No. 4, Spring Lot .....	200	0
Drilled Well at Open Reservoir (Distributing) .....	20	0
P.R.R., Parson's or Cases' Spring .....	10,000	24
Taps:		
Elmira Street .....	2,500	1
Canton " .....	1,500	1
Canton " .....	600	0
Troy Creamery .....	50,000	0
Penn. R.R. Station .....	12,000	0
Lower Elmira Street .....	24,000	0

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## DIAGNOSIS.

The epidemiologic diagnosis was based on the explosive character of the outbreak, the facts relative to the changes in the water system and the exclusion of milk, foods, ice, and ice cream as being improbable means of transmission. The individual diagnosis was in many cases more difficult to establish, especially early in the epidemic when a large percentage of the cases were in the prodromal stage of the disease. On the date of our arrival, physicians were not willing to report them as cases of typhoid fever to the local Board of Health because of the atypical onsets, and to a certain extent, atypical clinical courses. It was possible to study in consultation with the local physicians, a large proportion of the patients. From experience in previous epidemics in Pennsylvania it was advised that the sera of all atypical cases should be studied in relation to the following microorganisms: *B. typhosus*, *B. paratyphosus* A and B, *B. para-coli*, *B. enteritidis* (Gaertner), and *B. suisepiticus*. This work was performed by the State Laboratories without expense to the physicians or patient. A constantly negative agglutination was obtained with *B. suisepiticus*. In 47 cases the titer limit was studied in relation to each of the above microorganisms. Sixteen of these cases showed the presence of agglutinins for the *B. enteritidis* (Gaertner) and were negative to all other microorganisms throughout the course of illness in which so studied.

It is interesting in this relation to note the fact that the only pathogenic member of the typho-colon series which was recovered from the water-supplies was a form identical in its biochemical and iso-murtoric behavior with the original Gaertner form of *B. enteritidis*; this microorganism was recovered from samples of water collected for the purpose from the distributing reservoirs. The sera of the 16 patients referred to agglutinated this microorganism in dilutions of 1-50 at the end of one hour. For purposes of sanitary supervision, these 16 cases have been included in the total of 229.

## MORTALITY.

The mortality in this epidemic was 19, or 8.29 per cent. This is an unusually low mortality as in similar water-borne epidemics

in Pennsylvania the mortality usually ranges from 10 per cent to 14 per cent.

In other words, this epidemic presents a high case morbidity with a low case mortality. The first reasonable suggestion is that the particular strain of *B. typhosus* was of reduced virulence. Had the low mortality depended entirely upon diluted pollution, it is probable that the relative morbidity would have been lower, despite the apparent susceptibility of those exposed. While definite conclusions cannot be made, it is suggested that the particular bacillus, having passed 6 to 8 months in a host who had developed resistance to its pathogenic power, would undergo certain modifications in virulence.

#### TREATMENT.

Having established a tentative diagnosis on the day of arrival, measures, under the supervision of Mr. H. E. Moses, sanitary engineer of the State Department of Health, were taken to eliminate the infected waters, to furnish sufficient supply for fire protection and to establish a treatment plant. In order to accomplish this, it was necessary to cut out from service the Pennsylvania Railroad spring supply as well as the spring supply polluted by the creek waters. Because of the connection at the "Spring Lot" it was possible to continue the use of water from the springs and the drilled wells, and to this was added the supply from the drilled well at the distributing reservoirs.

The water in both reservoirs was treated with copper sulfate solution in the proportion of two parts per million gallons. This treatment was continued over a period of nearly two days, by which time a temporary plant for treating with hypochlorite of lime was put into service (Fig. 4). The distributing reservoirs were disinfected on October 16 and again on October 19, with copper sulfate solution and subsequently washed out with the treated water. At the same time the street mains were flushed, all dead ends and taps being opened until the water showed the presence of hypochlorite of lime at all points. The use of hypochlorite of lime has been continued to this date without the occurrence of any cases and pending the submission of plans by the engineer employed by the Borough.

After an examination of a large number of cases on the afternoon of October 13, the local Board of Health and physicians were advised to use antityphoid vaccine. This suggestion was immediately followed, and those who were not inoculated by their family physician were offered free vaccination at stations established by the Borough authorities. Physicians from neighboring points offered their services free of charge, and in co-operation with the local physicians and myself, 761 persons, or 56.66 per cent of the total population exposed to the infection, received the vaccine. Of these, 8, or 1 per cent, received inoculations conforming to the formula used in the United States Army; that is:

	Syringe A	Syringe B	Syringe C
<i>B. typhosus</i> .....	500	1,000	1,000 million

753, or 99 per cent received a trivalent vaccine in three doses, containing the following:

	Syringe A	Syringe B	Syringe C
<i>B. typhosus</i> .....	500	1,000	1,000 million
<i>B. paratyphosus</i> A.....	250	500	500 "
<i>B. paratyphosus</i> B.....	250	500	500 "

The inoculations were made at intervals of from eight to ten days.

A tabulation of the results is as follows:

Total number of persons exposed.....	1,343
" " " " vaccinated.....	761 or 56.66 per cent
" " " " unvaccinated.....	582 or 43.32 per cent

The study of the use of vaccine in relation to onsets and morbidity is as follows:

Total number of cases.....	229
Onsets prior to use of vaccine.....	127 or 55.46 per cent
" after the " " " .....	102 or 44.54 per cent

The morbidity in relation to onsets and use of vaccine shows that 65, or 63.72 per cent, were not inoculated, while 37, or 36.27 per cent, were inoculated.

The morbidity in relation to the total number of persons vaccinated and unvaccinated shows the following:

No. of persons vaccinated.....	761
" " these developing typhoid fever.....	37 or 4.86 per cent
" " persons not vaccinated.....	582
" " these developing typhoid fever.....	65 or 11.16 per cent

It will be noted from this table that 102 persons developed the onset of the disease subsequent to inoculation, and that 37, or 36 per cent, of the vaccinated and 65, or 64 per cent, of the unvaccinated developed the disease. It will also be noted that the infection of the water occurred (so far as it is possible to determine) prior to September 25, and was probably more or less continuously infected to October 7. The water was definitely disinfected on October 14, and it is reasonable to conclude that the incubation period of all persons exposed continued to about November 4. Hence, we have reason to believe that the use of antityphoid vaccine was an important factor in preventing the occurrence of a number of cases, and we are fortified in this opinion from the fact that this community, as has been noted above, has been a typhoid-free community, that the residents are of a sedentary rather than of an itinerant type, and have probably had less opportunity for gaining immunity through continued exposure in other places. Those who have traveled much traveled under conditions which made it improbable that they should be exposed to or develop an infection of this type.

Through the active co-operation of the local physicians and the ready acquiescence of every resident of the Borough, it was possible to establish prompt quarantine with isolation of every patient. Concurrent disinfection of all discharges, of all bed linens and clothing, of all utensils and the attendants' hands was efficiently carried out. Arrangements were made with the laundry in the Borough not to accept clothing or bed linens coming from known infected homes unless such pieces of laundry were first boiled and disinfected. All milk routes were placed under sanitary supervision, and no containers were permitted to be removed from premises in which the disease existed. Warning placards and circulars were distributed throughout the town, instructing the residents not to drink water which had not been boiled for at least 30 minutes. The same type of warning was printed in the local newspapers and in newspapers issued from nearby points but having a circulation in the Borough.

On October 16 an emergency hospital was opened and placed under the direction of the supervising nurses of the State Depart-

ment of Health. The patients admitted to this institution were carefully selected with reference to their physical condition, to the possibility or impossibility of carrying out proper sanitation, or to the financial condition of the family. There was a total of 49 admissions to this institution with a mortality of 4, or 8 per cent. For the most part the patients admitted were seriously ill. All cases remained under the charge of their own physician. The hospital was closed on December 12, 1912.

Through local philanthropic agencies and particularly through the offices of the local visiting nurses' organization, an efficient district nurse service was inaugurated at once. This was particularly valuable in premises where assistance was needed and yet for various reasons the patients could not be admitted to the emergency hospital. The work of the district nurse made it possible for each patient to be visited two or three times each day and for a nurse to remain on duty for long periods of time in cases where there was need of prolonged assistance.

A relief committee was promptly organized to help those who needed assistance, and contributions were received from many points in Pennsylvania and New York. This organization was placed under the management of a sub-committee, a station was opened, and a careful record was kept of all monies and other supplies received and distributed. From the first day of its organization no assistance was rendered without a hasty but careful investigation as to the need for such assistance. There is no doubt but that the relief from mental distress on the part of many of the wage-earners contributed to their eventual recovery without serious complications, and it would appear from the experience in Troy that such sociologic measures are an important factor in limiting an epidemic and in reducing the mortality.

The features of the treatment of this epidemic were found in the opportunities to carry out all of the modern methods in detail. The diagnosis was established within 24 hours after arrival and was followed by prompt engineering interference. For the first time (so far recorded) an antityphoid vaccine was generally used during the height of an epidemic. Through local, individual, municipal, and county co-operation an Emergency Hospital and District

Nurse Service, sanitary and educational measures, and financial assistance were applied with minimum delay and maximum results. The number of secondary cases is often a measure of preventive work done; in Troy there were but 4 secondary cases, 3 of which were nurses; the 4th case was in all probability, and according to the evidence collected, infected by a temporary "carrier" in the same premises. The advisability of having physicians, nurses, and other contacts receive immunization by means of a vaccine is emphasized.



# A SYSTEMATIC STUDY OF THE COCCACEAE IN THE COLLECTION OF THE MUSEUM OF NATURAL HISTORY.\*

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Five years ago the Winslows published a book<sup>1</sup> in which they proposed the adoption of the statistical method for the systematic classification of bacteria. They made a careful study of 500 strains of cocci which, together with the similar work on streptococci by Andrewes and Horder,<sup>2</sup> covered the entire family of the Coccaceae. It appeared that by applying a number of morphological and biochemical tests, recording the results quantitatively, wherever possible, and plotting them, the cocci grouped themselves about a few well-defined type centers. The correlation between different characters indicated that there are two main series or subfamilies among the Coccaceae, one primarily of parasitic origin and the other commonly found in water and earth outside the body. Within these two subfamilies, subgroups appeared conveniently characterized by the chromogenesis of their growth on agar. The various physical and physiological properties of the organisms correlated so well with that of pigment production that genera could be broadly distinguished in accordance with this character; and a natural biological grouping was apparently obtained.

Since the appearance of this book various workers have applied the same principle to other groups of bacteria. Winslow,<sup>3</sup> Broadhurst,<sup>4</sup> Stowell and Hilliard<sup>5</sup> have attacked the difficult problem of discovering some order among the streptococci with promise of success. Howe<sup>6</sup> has applied this method in a study of the colon group. Morse<sup>7</sup> has recently published the results of a very successful application of the principles of biometry to the study of the

\* Received for publication February 27, 1913.

<sup>1</sup> *Systematic Relationship of the Coccaceae*, 1908.

<sup>2</sup> *Lancet*, 1906, 2, p. 708.

<sup>3</sup> *Jour. Infect. Dis.*, 1912, 10, p. 285.

<sup>4</sup> *Ibid.*, 1912, 10, p. 272.

<sup>5</sup> *Science* 1912, 35, p. 223.

<sup>6</sup> *Ibid.*, 1912, 35, p. 225.

<sup>7</sup> *Jour. Infect. Dis.*, 1912, 11, p. 253.

diphtheria group. No one has, however, thought it desirable to test, verify, and possibly extend, the results obtained by the Winslows, in regard to the group of the cocci as a whole.

The large number of organisms in our bacterial museum and the facilities thus afforded for systematic work, led me, at the suggestion of Professor Winslow, to undertake a biometric study of the cocci in our collection. The cocci, other than those belonging to the streptococcus and diplococcus groups, numbered 54. These strains were sent to us under 30 different names, representing, according to the classification of Migula and Chester, as many distinct species. It was interesting to see, therefore, whether the biometric classification could be applied to this large assortment of species, and how these supposedly distinct types would group themselves. The results speak for themselves.

The 54 strains in our collection representing the various groups of cocci were put through a series of 10 of the tests found by the Winslows to be of classificatory value. In addition to these, the acid production in a 1 per cent solution of saccharose broth and the ammonification of a 1 per cent solution of peptone were observed. The value of the former test could not be discerned in the small number of organisms tested (in general it behaves similarly to the other disaccharid, lactose), while the latter proved to be highly important.

The methods used were, with slight modifications, those followed by the Winslows. As a detailed discussion is very clearly presented by them, a concise statement of the tests with some brief comments, where necessary, will here suffice.

1. *Habitat*.—Unfortunately only 20 odd strains have a definite history, as to the source of isolation. Where the origin is known, the white and orange forms are generally from the human or animal body, while the yellow and red forms are from air, water, or other saprophytic sources.

2. *Grouping and dimensions*.—Observed on stained specimens from agar streaks after 3 or 4 days at 20° C. and 37° C. respectively. Recorded as: (1) packets, (2) no packets. The results were checked on another occasion from young agar streaks at 20° C.

3. *Gram reaction*.—Observed on three-days-old agar streaks at 20° C. and 37° C. on two different occasions.

4. *Vigor of surface growth*.—After 14 days on agar at 20°. Recorded as (a) faint; (b) meager; (c) good; (d) abundant; (e) very abundant.

5, 6, 7. *Acid produced*, after 14 days at 20° in 1 per cent solutions of dextrose, lactose, and saccharose broth. Determined by titration with N/20 NaOH, using phenolphthalein as indicator and titrating and subtracting value of controls. Results recorded in percentage normal.

8. *Nitrite and ammonia* production in nitrate broth, tested in the usual way, after incubating for 14 days at 20° C. Ten tubes were used for each strain. About 25 of the cultures were repeated, incubating for only seven days. No appreciable difference was found, except that in a few instances the intensity of the reaction (hence the amount of the ammonia or nitrite) was decreased. In some cases, notably among the micrococci, ammonia only was produced, and repeated tests failed to show the presence of nitrites. Winslow<sup>1</sup> obtained similar results and was led to believe that this was a case of direct reduction of nitrates to ammonia. It seemed probable, however, that the ammonia was the product of the breaking down of the peptone present in the nitrate broth. This assumption was carefully tested and proved to be correct. The results of this study were reported at the September meeting of the American Public Health Association, before the Laboratory Section. None of the organisms tested seemed to possess the power of breaking down nitrates to ammonia in a nitrate-peptone solution, the ammonia being derived entirely from the peptone. The production of ammonia from peptone proved to be a distinct and valuable test.

9. *Production of ammonia* in nitrate-free peptone broth was tested by inoculating each organism into five tubes containing a solution of 1 per cent peptone in distilled water to which some inorganic salts were added. These tubes were incubated for seven days at 20° C. The presence of ammonia was tested in the usual way by Nesslerization and the results were recorded as positive or negative. The value of this test is brought out by a correlation of the pigment-producing property with that of ammonification of peptone as shown in Table 1.

<sup>1</sup> *Systematic Relationship of the Coccaceae*, 1908.

TABLE 1.  
CORRELATION BETWEEN CHROMOGENESIS, REDUCTION OF NITRATE, AND AMMONIFICATION  
OF PEPTONE

CHROMOGENESIS	TOTAL NUMBER OF STRAINS	REDUCTION OF NITRATE TO NITRITE		SPLITTING OF PEPTONE TO AMMONIA	
		Number	Percentage	Number	Percentage
None.....	6	0	0	0	0
White.....	12	1	8.3	4	33.3
Orange*	15	11	73.3	14	93.3
Yellow.....	15	2	13.3	12	80.0
Red.....	5	5	100.0	1	20

\* Including the orange sarcinae.

10. *Comparative growth* after 14 days at 20° and 37° respectively. Recorded as: (a) better at 37°; (b) same; (c) poorer at 37°.

11. *Chromogenesis*.—The color of the pigment produced on agar streaks, after 14 days at 20° C. A loopful of the growth was spread in a thin uniform layer on white paper and dried at room temperature. This was then compared with the chart given as the frontispiece of the Winslows' book on the Coccaceae.<sup>1</sup> It is essential that an approximately equal amount of growth be used and that this be spread evenly on the paper. This is especially important in the case of the meager-growing orange and white cocci. In these organisms the pigments, when observed on the brownish agar background, can very readily be confused. As will be seen from the table below, at least one typical aurococcus (No. 218) was sent to us as an albococcus. The distinction is quite sharp, when the growth is spread and examined on paper. The chromes, using the chart in the Winslows' book, fall as follows:

*White*.—white column; light lemon yellow I-III; cadmium yellow I-III.

*Yellow*.—cadmium yellow IV-IX; medium cadmium yellow column; orange yellow I-IV.

*Orange*.—orange yellow V-IX; cadmium orange column.

*Red*.—orange red; medium red, dark red.

12. *Gelatin liquefaction*.—Recorded in centimeters after 30 days at 20° C., in tubes about 1.1 cm. in diameter.

<sup>1</sup> *Op. cit.*

TABLE 2.

No. of Culture	SOURCE	ORIGINAL NAME	GROUPING OF CELLS	DIMENSION IN $\mu$	GRAM REACTION	GROWTH AFTER 14 DAYS AT		ACID PRODUCTION PERCENTAGE NORMAL IN			REDUCTION OF NITRATES TO NITRITES	SPLITTING OF PEPTONE TO AMMONIA	LIQUEFACTION NO. OF CMS.	CHROMO-GENESIS	NEW NAME
						20° C.	37° C.	Dext.	Lact.	Sac.					
3...	U. of Penn.	<i>S. aurantiaca</i>	Packets	0.9	—	Abundant	Poorer	1.4	-0.9	-0.4	+	+	2.6	Orange	<i>S. aurantiaca</i>
4...	Rockefeller Inst.	<i>M. mellensis</i>	No packets	0.7	—	Meager	Better	2.0	2.8	4.4	+	+	2.7	Orange	<i>Aur. mollis</i>
*33...	From arm vein, U. of C.	"	"	0.4	—	Meager	Same	-0.1	-0.2	0.0	+	+	0.0	Orange	<i>B. melitensis</i>
34...	Rockefeller Inst.	<i>M. rheumaticus</i>	"	0.8	—	Faint	Same	3.6	2.8	0.5	—	—	0.0	None	<i>Str. rheumaticus</i>
101...	Lab. air. Del. Ag. Col.	<i>Sarcina</i>	Packets	0.8	—	Abundant	Same	0.0	0.0	0.3	—	+	1.4	Yellow	<i>S. flava</i> (Var. B)
113...	Air contamination, U. of Pa.	<i>S. flava</i>	"	0.6	—	"	Poorer	1.2	-0.3	1.2	—	+	2.2	Yellow	<i>S. flava</i> (Var. B)
128...	Cespool, U. of C.	<i>M. zymogenes</i>	No packets	0.8	—	Faint	Same	6.0	2.8	0.7	—	—	4.5	None	<i>Str. gracilis</i>
130...	Urine, Mt. Prospect Lab.	<i>M. ureae</i>	"	0.6	+	Good	Poorer	1.9	2.0	2.7	—	+	1.1	White	<i>Alb. ureae</i>
174...	U. of C.	<i>S. capsulata</i>	"	0.6	+	Good	Same	2.1	2.8	3.1	—	+	0.0	White	<i>Alb. candidus</i>
207...	Subcutaneous abscess, U. of Iowa	"	"	0.6	+	Meager	Better	3.0	2.8	3.5	+	+	4.7	Orange	<i>Aur. mollis</i>
208...	Stomach contents of chronic dyspepsia, U. of Iowa	<i>S. lutea</i>	Packets	0.6	+	Very abundant	Poorer	0.3	0.0	0.3	—	+	2.8	Yellow	<i>S. flava</i> (Var. A)
209...	Abscess in calf of leg, U. of Iowa	<i>M. tetragenus</i>	Tetrads	0.6	+	Meager	Same	1.9	3.0	2.3	—	—	0.0	White	<i>Alb. tetragenus</i>
216...	Tap water, J.H.U.	<i>S. lutea</i>	Packets	0.7	+	Very abundant	Poorer	1.6	0.3	1.0	—	+	4.7	Yellow	<i>S. flava</i> (Var. B)
217...	J.H.U.	<i>Albococcus</i>	No packets	0.8	—	Abundant	Same	0.5	-0.2	9.0	+	+	0.0	Red	<i>Rh. roseus</i>
218...	J.H.U.	"	"	0.8	+	Meager	Same	2.8	1.0	2.4	+	+	4.7	Orange	<i>Aur. mollis</i>
219...	J.H.U.	"	"	0.7	+	Meager	Better	2.8	2.6	4.3	+	+	0.0	Orange	<i>Aur. mollis</i>
220...	J.H.U.	<i>M. cereus</i>	"	0.7	+	Abundant	Poorer	-0.8	-0.8	-0.3	—	—	0.0	Yellow	<i>M. luteus</i>
260...	P.D. & Co.	<i>M. neoformans</i>	"	0.7	+	Abundant	Same	1.8	0.4	0.6	—	—	0.0	White	<i>Alb. candidus</i>
261...	Abscess of horse, P.D. & Co.	<i>Str. pyogenes</i>	"	0.7	—	Good	Same	1.2	-0.3	0.6	—	—	0.0	White	<i>Alb.</i>
262...	Abscess of horse, P.D. & Co.	<i>Str. pyogenes</i>	"	0.6	+	Meager	Same	2.7	2.0	2.6	+	+	2.2	Orange	<i>Aur. mollis</i>
263...	Skin lesion, P.D. & Co.	<i>Str. pyog. albus</i>	"	0.8	+	Meager	Better	1.8	1.7	2.7	—	+	1.0	White	<i>Alb. pyogenes</i>
264...	Bulla, P.D. & Co.	<i>Str. pyog. aureus</i>	"	0.7	+	Meager	Better	2.5	2.8	4.0	—	+	3.2	Orange	<i>Aur. mollis</i>
265...	P.D. & Co.	<i>M. rheumaticus</i>	"	0.8	—	Faint	Same	6.8	4.3	7.2	—	+	0.0	None	<i>Str. rheumaticus</i>
272...	P.D. & Co. (Kral)	<i>Str. cereus flavus</i>	"	0.7	—	Very abundant	Poorer	0.0	0.4	0.8	—	+	2.3	Yellow	<i>M. flavus</i>
273...	"	"	"	0.7	+	ant	Better	1.5	2.5	3.8	+	+	4.3	Orange	<i>Aur. mollis</i>
279...	Acne vulgaris, P.D. & Co.	<i>Str. pyogenes citreus</i>	"	0.7	+	Meager	Better	4.0	2.2	3.2	+	+	2.8	Orange	<i>Aur. mollis</i>
312...	Mammary abscess, Boston Board of Health	"	"	0.7	+	Meager	Better	4.0	2.2	3.2	+	+	2.8	Orange	<i>Aur. mollis</i>

\* Repeated stains of cultures of different ages showed this to be a small bacillus, 4X.5  $\mu$ .

TABLE 2—Continued.

[illegible]

A complete record of all the strains studied, giving in detail the history of each culture (as far as possible), the original name under which it was received, the various tests recorded as observed, and finally the new name according to the Winslow classification, is shown in Table 2. This table points out very clearly the necessity of a definite system of classification based on real differences and definite properties. We see here a list of organisms, some of which (like 217, 462, and 483), differ in nothing but in the name under which they were sent to us; others (like 218 and 279) are not really what they were supposed to be. This can be attributed to nothing else than the poverty of the traditional descriptions, inevitable under a system of differentiation based on such variable factors as surface growth on various media, types and contour of colonies, etc.

On breaking up this table and grouping the organisms according to the general correlation of their properties, we obtain five main groups agreeing in all essentials with the genera defined by the Winslows. Table 4 shows a group of non-pigment producing, high acid forming, faintly growing organisms. These belong undoubtedly to the genera *Streptococcus* and *Diplococcus* as defined by the Winslows. The white-pigment producers grouped in Table 5 answer well to the characteristics of the genus *Albococcus* (Winslow and Rogers). The essential characters of the group, such as generally gram-positive, good growth on agar, moderate acid production in all three sugars, slight gelatin liquefaction and nitrate reduction, correspond with those attributed to this genus.

The orange cocci, as seen from Table 6, also present, in accordance with the findings of the Winslows, a distinct and definite picture. These organisms generally stain by Gram, give a meager growth on agar, ferment all the sugars tested, actively liquefy gelatin, and generally reduce nitrates. This group agrees very well with the genus *Aurococcus* (Winslow and Rogers).

In Table 7 (*a* and *b*) are included all the strains producing yellow pigment. This table brings out very strikingly the fact that there is practically no difference between the yellow micrococci and sarrinae other than that of cell grouping. The value of packet formation as a generic differential seems extremely doubtful. In accord-

ance with the Winslows, however, this group is divided into two parts: (a) corresponding to the genus *Micrococcus* (Hallier, Cohn) and (b) to the genus *Sarcina* (Goodsir). The group as a whole presents a definite unit and agrees well with the Winslows' definition. It generally decolorizes by Gram; gives abundant growth on agar; slight acid in dextrose; a generally neutral reaction in lactose; while gelatin is frequently liquefied.

The last group, consisting of the red-pigment producers, is shown in Table 8. Like the others, it corresponds very closely to its respective genus *Rhodococcus* (Winslow and Rogers). It is generally gram-negative and gives good to abundant surface growth. Sugars are but slightly fermented. Gelatin is rarely liquefied. Nitrates are generally reduced.

TABLE 3.  
CORRELATION OF CHARACTERS OF THE DIFFERENT GENERA.

CHROMO-GENESIS	GENUS	GROUPING OF CELLS; PERCENTAGE PACKETS	GRAM REACTION; PERCENTAGE NEGATIVE	SURFACE GROWTH; PERCENTAGE GIVING				ACIDITY; PERCENTAGE ABOVE 0.01 N.			NITRATE REDUCTION; PERCENTAGE REDUCERS	PEPTONE AMMONIFICATION; PERCENTAGE AMMONIFIERS	GELATIN LIQUEFACTION; PERCENTAGE LIQUEFIERS	GELATIN LIQUEFACTION; AVERAGE IN CM.
				Faint	Mesiger	Good	Abundant	Dextrose	Lactose	Saccharose				
None.....	<i>Streptococcus</i> and <i>Diplo-</i> <i>coccus</i>	0	66	100	0	0	0	100	100	66	0	0	17	...
White.....	<i>Albococcus</i>	0	17	0	25	75	0	100	75	85	0	33	25	1.4
Orange*.....	<i>Aurococcus</i>	20	7	0	73	7	20	100	80	80	73	93	87	3.5
Yellow.....	<i>Micrococcus</i>	0	87	0	0	0	100	12	0	0	25	87	63	2.2
Yellow.....	<i>Sarcina</i>	100	71	0	0	0	100	20	0	0	0	71	100	2.3
Red.....	<i>Rhodococcus</i>	0	80	0	0	80	20	0	0	0	100	20	20	0.0

\* Includes orange *sarcinae*.

The generic classification proposed by the Winslows is thus found to apply easily and satisfactorily to the 54 strains of cocci in our collection. While there may be now and then an aberrant strain, the remarkable general correlation between pigment production and other properties justifies the grouping of the cocci into the genera named above.

A summary of the correlation of the various characters of the different generic groups is given in Table 3. The comparatively small number of organisms in each group leads often to percentages



which are either too high or too low; and it is of course obvious that the classification is not a sharp nor absolute one. For instance, the yellow sarcinae are characteristically gram-negative, yet 29 per cent of the strains studied showed a positive reaction. Bacteria are exceedingly variable organisms and this variability is indeed the main reason for the use of the biometric method. The general agreement which exists between the results tabulated below and those obtained in Boston<sup>1</sup> five years ago is too close to be accounted for except by an inherent, though not infallible, correlation between the various properties considered; and such a correlation can only be explained on a basis of genetic relationships.

On analyzing each of the main groups carefully we find that the classification of species proposed by the Winslows is not quite as satisfactory as their generic grouping. There appear to be certain forms which the authors have not found among the organisms studied by them but which are important enough to deserve specific rank. This is exactly what might have been expected and can be remedied only by further and more elaborate study. The behavior of the cocci in splitting up peptone, which is here used for the first time, of course, increases the possibility of defining new and distinct species.

Of the strains grouped in Table 4, No. 128 undoubtedly belongs to the species *Str. gracilis* of which *M. zymogenes* is a synonym. Nos. 34, 259, and 399, sent to us as *M. rheumaticus*, may for the present be retained as *Str. rheumaticus*, since their behavior (faint growth, high acid production, and negative reaction in other media) corresponds to that of the genus *Streptococcus*, while they have nothing in common with the micrococci. Whether this group is distinct enough to deserve specific rank or not is still an open question to be decided by a comparative study of a large number of strains of this type. In a recent paper Major<sup>2</sup> has reported a more detailed examination of the fermentation reactions of these three strains and finds that No. 34 belongs to the *S. salivarius* while Nos. 259 and 399 are examples of the *S. fecalis* of Andrewes and Horder. No. 437 was sent to us as *M. catarrhalis*. This coccus is defined

<sup>1</sup> Winslow, C.-E. A and A. R., *Systematic Relationship of the Coccaceae*.

<sup>2</sup> *Johns Hopkins Hosp. Bull.*, 1912, 23, p. 326.

TABLE 4.  
NO PIGMENT PRODUCERS

No. of CULTURE	ORIGINAL NAME	GROUPING OF CELLS	DIMENSION IN $\mu$	GROWTH AT		GRAM REACTION	ACID PRODUCTION IN			NITRATE REDUC- TION TO NO <sub>2</sub>	BREAKING DOWN OF PEPTONE TO NH <sub>3</sub>	GELATIN LIQUOR- REACTION	NEW NAME
				20° C.	37° C.		Dext.	Lact.	Sacch.				
128.....	M. symogenes	Short chains	0.8 $\mu$	Faint	Same	+	++++++	++++++	++++++	++++++	++++++	++++++	Sir. gracilis
34.....	M. rheumaticus	Irregular groups and small chains	0.8 "	"	"	+	++++++	++++++	++++++	++++++	++++++	++++++	Sir. rheumaticus
259.....	M. rheumaticus	Irregular groups, short chains	0.8 "	"	"	+	++++++	++++++	++++++	++++++	++++++	++++++	"
309.....	M. rheumaticus	Irregular groups, short chains	0.8 "	"	"	+	++++++	++++++	++++++	++++++	++++++	++++++	"
437.....	M. catarrhalis	Short chains	0.7 "	"	"	+	++++++	++++++	++++++	++++++	++++++	++++++	Sir. ?
476.....	?	Irregular groups, short chains	0.8 "	"	"	+	++++++	++++++	++++++	++++++	++++++	++++++	Sir. ?

by Frosh and Kolle<sup>1</sup> as gram-negative, and was found by Elser and Huntoon<sup>2</sup> to be gram-negative and non-fermenting. Our strain is gram-positive and ferments sugars actively. It cannot, therefore, belong to this species. Its actual place, as well as that of No. 476, must be left open for the present, though both of them undoubtedly belong to the genus *Streptococcus*.

No attempt has been made to include the large number of streptococci in the Museum collection in this study. The six forms mentioned above came to us as micrococci and were classed as such until this examination was made. This illustrates clearly how, under the present system of classification, totally unrelated organisms are included in the same genus.

In the white-pigment producing group (see Table 5) we find that No. 263 corresponds to the species *Alb. pyogenes* (Rosenbach) Winslow, in that it liquefies gelatin and does not reduce nitrates. Nos. 464 and 130 are, like 263, liquefiers and non-reducers, but unlike that strain they possess the power of breaking down peptone to ammonia. The Winslows do not recognize this as a distinct species, yet those two organisms differ from the nearest type (*Alb. pyogenes*), in the important properties of growth abundance and formation of ammonia from peptone. Both organisms were sent to us as *M. urea*, and urea-fermenting organisms having the power of producing ammonia are described by many observers. Flügge<sup>3</sup> defines a white, urea-fermenting, gelatin-liquefying coccus as *M. Ureae-liquefaciens*. It seems justifiable, therefore, to recognize this type and characterize it as follows: *Alb. ureae* (Cohn, Flügge), a white coccus occurring singly or in irregular groups, generally found in the human or animal body. Generally gram-positive. Good surface growth. Produces moderate acid in dextrose, saccharose, and lactose media. Gelatin liquefied. Nitrate not reduced. Peptone decomposed to ammonia.

Nos. 484 and 209 (Table 5) evidently belong to the species *Alb. tetragenus* (Cohn) Winslow. This type is a non-reducer and a non-liquefier and possesses the characteristic grouping of cells in

<sup>1</sup> *Die Mikrokokken*. Flügge's *Die Mikroorganismen*, 1896, Vol. 2.

<sup>2</sup> *Jour. Med. Research*, 1909, N. S. 15, p. 413 and p. 427.

<sup>3</sup> *Die Mikroorganismen*, 1896, Vol. 2, p. 173.

TABLE 5.  
WHITE PIGMENT PRODUCERS.

No. of Culture	ORIGINAL SOURCE	ORIGINAL NAME	GROUPING OF CELLS	DIMENSION IN $\mu$	GRAM REACTION	GROWTH AT		ACID PRODUCTION IN			BREAKING DOWN OF PEPTONE TO $\text{NH}_3$	REDUCTION OF NITRATE TO $\text{NO}_2$	GELATIN LIQUEFACTION	NEW NAME
						20° C.	37° C.	Dext.	Lact.	Sacch.				
263...	Pathogenic	<i>St. pyogenes albus</i>	No packets	0.8	++	Meager	Better	++	++	++	++	++	++	<i>Alb. pyogenes</i>
130...	Human	<i>M. ureae</i>	"	0.6	++	Good	Poorer	++	++	++	++	++	++	<i>Alb. ureae</i>
464...	"	"	"	0.6	++	Good	Same	++	++	++	++	++	++	<i>Alb. ureae</i>
209...	Pathogenic	<i>M. tetragenus</i>	tetrads	0.6	++	Good	Same	++	++	++	++	++	++	<i>Alb. tetragenus</i>
484...	"	"	"	0.6	++	Good	Better	++	++	++	++	++	++	"
174...	Pathogenic	<i>S. capsulata</i>	No packets	0.6	++	Good	Same	++	++	++	++	++	++	<i>Alb. candidus</i>
471...	"	<i>M. candidans</i>	"	0.7	++	Good	Poorer	++	++	++	++	++	++	"
525...	Pathogenic	<i>M. acne</i>	"	0.7	++	Meager	Same	++	++	++	++	++	++	"
467...	"	<i>M. aurantiacus</i>	"	0.9	++	Good	Same	++	++	++	++	++	++	<i>Alb. epidermidis</i> (Var. A)
260...	"	<i>M. neofornans</i>	"	0.7	++	Good	Same	++	++	++	++	++	++	<i>Alb. candidans</i>
261...	Horse abscess	<i>St. pyog. albus</i>	"	0.7	++	Good	Same	++	++	++	++	++	++	"
526...	"	<i>M. neofornans</i>	"	0.7	++	Good	Same	++	++	++	++	++	++	"

tetrads. Nos. 174, 471, and 525 agree with the definition of *Alb. candidus* (Cohn) Winslow, in failing to reduce nitrates or liquefy gelatin. No. 174 came to us as a sarcina which it is not. No. 467 came to us as *M. aurantiacus*, but is not an orange-pigment former. It apparently finds no place in the Winslows' classification. The authors call this a rare aberrant type, which may be termed a "variant by suppression" of the reducing and liquefying type which has lost the latter property. Though this one strain does not justify the creation of a new type center, yet the recurrence of similar *non-liquefying* and *reducing* types in the other groups tends to point to the possible existence of such a type center. Temporarily it may be called *Alb. epidermidis* (Var. A). Nos. 260, 261, and 526 are even more puzzling. These seem to occupy a position between the albococci and micrococci. They differ from the albococci mainly in their lack of power to ferment the disaccharids. Winslow mentions this species as the second of Gordon's four types of skin cocci and places it among the micrococci as *M. candicans*. From the work of Gordon it appears, however, that they are body forms. Their relation to the albococci in gram reaction, pigment production, growth abundance and fermentation of dextrose is closer than to the micrococci. It appears, therefore, more consistent to place them in this group. This question may perhaps best be left open until more work has been done; but for the present I suggest the name *Alb. candicans* rather than *M. candicans*, for the type of white coccus which acidifies dextrose but not lactose and fails to reduce nitrates or liquefy gelatin. It is interesting to note that two of these three cultures were sent to us under the name *M. neoformans*, the coccus isolated from cancerous tissue by Doyen.

Examining the third group (see Table 6), we find that No. 347 in liquefying gelatin and not reducing nitrates answers to the type *Aur. aureus* (Rosenbach) Winslow. *Aur. mollis* (Dyar) Winslow, the reducing and liquefying type, is represented in our collection by Nos. 218, 219, 262, 264, 4, 207, 279, 312, 348. Nos. 313 and 457, reducers but slow liquefiers, are aberrant types similar to No. 467 among the albococci. For similar reasons these may be classed as a variety of the reducing and liquefying species *Aur.*

TABLE 6.  
ORANGE PROMENT PRODUCERS.

No. of Culture	Original Source	Original Name	Grouping of Cells	Dimension in $\mu$	Gram Reaction	Growth at		Acid Production in			Splitting of Peptone to $\text{NH}_3$	Reduction of Nitrate to $\text{NO}_2$	Gelatin Layer	New Name
						20° C.	37° C.	Dext.	Lact.	Sacch.				
347	Parasitic	St. pyogenes	No packets	0.7	+	Meager	Better	+	+	+	+	+	Aur. aureus	
4	.....	.....	"	0.7	+	"	"	+	+	+	+	+	Aur. mollis	
207	Parasitic	St. pyogenes aureus	"	0.6	+	"	Same	+	+	+	+	+	"	
202	"	Albococcus	"	0.6	+	"	Better	+	+	+	+	+	"	
218	.....	.....	"	0.7	+	"	"	+	+	+	+	+	"	
219	Parasitic	St. pyogenes aureus	"	0.7	+	"	"	+	+	+	+	+	"	
204	"	St. pyogenes citreus	"	0.7	+	"	"	+	+	+	+	+	"	
279	"	.....	"	0.7	+	"	"	+	+	+	+	+	"	
312	.....	.....	"	0.7	+	"	"	+	+	+	+	+	"	
348	Parasitic	St. pyogenes	"	0.8	+	Good	Little	+	+	+	+	+	Aur. Mollis (Var. A)	
313	"	.....	"	0.8	+	Meager	Poorer	+	+	+	+	+	"	
457	"	M. asciformans	"	0.6	+	"	Same	+	+	+	+	+	"	
3	.....	S. aurantiaca	Packets	0.9	+	Abundant	Better	+	+	+	+	+	Sarcina aurantiaca	
315	.....	"	"	0.8	+	"	Poorer	+	+	+	+	+	"	
474	.....	.....	"	0.9	+	"	"	+	+	+	+	+	"	

*mollis*. It may be noted that No. 457 came to us under the name *M. ascoformans*.

Strains 3, 315, and 474, though producing a strong orange color, present a picture entirely different from that of the aurococci. The generally abundant growth, frequent decolorization by Gram (more often than aurococci), better growth at 20°, lack of fermentative power of lactose and saccharose, slower liquefaction of gelatin, together with the formation of packets, bring this type closer to the yellow sarcinae than to the aurococci. It differs from the yellow sarcinae, however, in its orange pigment and in attacking dextrose. The Winslows state that the 11 strains of orange-packet formers with which they worked were generally gram-positive, acid-forming, actively liquefying forms, and they are inclined to refer them to the genus *Aurococcus*. The three strains here studied seem, however, to be much more closely related to the genus *Sarcina* and will be considered further under that head.

Another form studied in this group was No. 33, the *M. melitensis* of Bruce. It is a meager, gram-negative, orange-pigment former, giving negative reactions in all media. A careful examination of our strain, however, confirms the conclusion of Babes<sup>1</sup> that this organism is a small bacillus.

Examining the micrococcus group shown in Table 7a, No. 466 represents the liquefying, reducing type defined as *M. citreus* (Dyar) Winslow. No. 479 in its failure to liquefy gelatin represents another of the aberrant types discussed before and may be classed as variety A of *M. citreus*. This organism came to us as a sarcina. Though no packets could be observed, its cultural characters make it appear probable that it is related to the *S. ventriculi* of Goodsir. Nos. 272, 354, 458, and 481 belong to the non-reducing, liquefying type *M. flavus* (Flügge) Migula. This type seems also to be characterized by the property of splitting peptone to ammonia. Whether this character is specific or not can be brought out only by further comparative study of the behavior of the cocci in this respect. No. 430 is grouped with 220 as *M. luteus* (Cohn) Migula, the non-reducing, non-liquefying type, although it differs from it in breaking down peptone to ammonia.

<sup>1</sup> Kolle and Wasseman, *Handbuch der pathogenen Mikroorganismen*, 1903, 3, p. 438.

TABLE 7.  
YELLOW PIGMENT PRODUCERS.  
(a) NON-PACKET FORMERS.

No. of Culture	Original Source	Original Name	Grouping of Cells	Dimension in $\mu$	Germ Reaction	Growth at	Acid Production in			Breaking Down of Peptone to $\text{NH}_3$	Reduction of Nitrate to $\text{NO}_2$	Gelatin Liquefaction	New Name
						20° C.	37° C.	Dext.	Lact.	Sacch.			
466....	.....	<i>M. agilis</i> (citreus)	No packets	0.6	-	Abundant	Poorer	-	-	-	+	+	<i>M. citreus</i> (Var. A)
479....	.....	<i>S. ventricula</i>	"	0.9	-	Very abundant	"	-	-	-	+	+	<i>M. flavus</i>
472....	.....	<i>Staph. cereus flavus</i>	"	0.7	-	Abundant	"	+	-	-	-	-	"
354....	Butter	<i>M. citreus</i>	"	0.9	-	"	"	-	-	-	+	+	"
458....	.....	"	Some tetrads	0.9	-	"	"	-	-	-	+	+	"
481....	.....	<i>S. flava</i>	No packets, some tetrads	0.9	-	Very abundant	"	-	-	-	-	-	"
220....	.....	<i>M. cereus</i>	No packets	0.8	-	Abundant	"	-	-	-	+	+	"
430....	.....	<i>M. versatilis</i>	No packets, some tetrads	0.7	-	"	"	-	-	-	-	-	<i>M. luteus</i>
				0.6	-	"	"	-	-	-	-	-	"

(b) PACKET FORMERS.

101....	Air	<i>Sarcina</i>	Packets	0.8	-	Abundant	Same	-	-	-	+	+	<i>S. flava</i>
345....	.....	<i>S. lutea</i>	"	0.6	-	Very abundant	Poorer	-	-	-	-	-	"
208....	Stomach content	"	"	0.6	-	"	"	-	-	-	-	-	"
314....	.....	"	"	0.8	-	"	"	-	-	-	-	-	"
482....	.....	<i>S. mobilis</i>	"	0.9	-	Abundant	"	-	-	-	-	-	"
113....	Air	<i>S. flava</i>	"	0.6	-	Very abundant	"	+	+	+	+	+	"
210....	Water	<i>S. lutea</i>	"	0.7	+	"	"	+	+	+	+	+	"
3....	.....	<i>S. aurantiaca</i>	"	0.9	-	Abundant	"	+	+	+	+	+	<i>S. aurantiaca</i>
315....	.....	"	"	0.9	-	"	"	+	+	+	+	+	"
474....	.....	"	"	0.8	-	"	"	+	+	+	+	+	"



In Table 7b—the *Sarcina* group—101 and 345 belong to the liquefiers and non-reducers, which are classed under the type *S. flava* (De Bary). Nos. 113, 216, 208, 314, 482 are all similar to *S. flava* in that they do not reduce nitrates but liquefy gelatin. They all differ from that form in their power to break up peptone to ammonia. Though not justified in recognizing a new type center, yet the comparatively large percentage of our sarcinae which fall in this group indicates at least the possible existence of such a species. Temporarily, they may be classed, therefore, as a variety of *S. flava*. No. 482 came to us as *S. mobilis*, supposed to be a motile red-pigment former, which it is not. Two forms, 113 and 216, of this subgroup show a further remarkable distinction in being able to ferment dextrose and saccharose. This points to a close relationship between these forms and the orange sarcinae. It is highly probable that these are intermediary forms between the yellow and orange sarcinae and possibly between the micrococci and the aurococci. If these forms occur frequently they certainly deserve specific recognition. Here again, however, two strains do not justify the establishing of a new type center. They are, therefore, for the present, classed as another variety (Var. B) of *S. flava*. A further study of this whole group is necessary and highly desirable.

Nos. 3, 315, and 474 remain to be considered. These are the orange sarcinae, which, as pointed out above, seem more nearly allied to the yellow sarcinae than to the aurococci. These three strains were all sent to us as *S. aurantiaca*, and all liquefy, corresponding with Flügge's description of that species. The type may therefore bear the name commonly given to it with the following definition:

*S. aurantiaca* (Flügge).—A large saprophytic coccus. Occurs in packets. Variably affected by Gram. Abundant orange growth. Reaction in dextrose broth moderately acid, in lactose and saccharose faintly alkaline. Gelatin liquefied. Nitrates not reduced. Peptone ammonified.

The definition of the species of the last genus proposed by the Winslows, *Rhodococcus*, is not quite satisfactory. This is undoubtedly due to the small number of strains studied by the authors. According to their classification all the strains grouped in Table 8

TABLE 8.  
RED PIGMENT PRODUCERS.

No. of Culture	Original Source	Original Name	Grouping of Cells	Dimension in $\mu$	Gram Reaction	Growth at		Acid Production in			Breaking Down of Peptone to $\text{NH}_3$	Reduction of Nitrate to $\text{NO}_2$	Gelatin Liquor-Factor	New Name
217....	.....	.....	No packets	0.8	+++	20° C.	37° C.	Dext.	Lact.	Sacch.		+++++	++++	Rhod. roseus
462....	.....	Sarcina rosea	"	0.7	++	Abundant	Same	----	----	----		+++++	++++	"
480....	.....	M. rhodochrous	"	0.6	++	Good	Poorer	----	----	----		+++++	++++	"
483....	.....	M. roseus	"	0.9	++	"	"	----	----	----		+++++	++++	"
488....	.....	M. agilis	"	0.9	++	"	"	----	----	----		+++++	++++	R. roseus (Var. A)

belong to the species *R. roseus* (Flügge, Dyar) Winslow. Strains 217, 462, and 483 are undoubtedly representatives of this species. No. 480 possibly also belongs here, though it was the only form that gave repeatedly a gram-positive reaction. No. 488, however, appears to be out of place in this group. Its pigment is of a deeper hue than that of any of the others (Nos. 217, 463, and 483 all fall in the first three [lighter] hues of the orange red of the Winslows' chart, while No. 488 falls in hue 5 of the dark red); it liquefies gelatin and shows a tendency to break up peptone to ammonia. It is as different from the others as one species can be from another. It may also be observed that this organism was sent to us as *M. agilis*. No motility could be observed however. The type is not defined by the Winslows, and one strain hardly justifies such a definition. We must resort again, therefore, to the distinction by variety and call this form *R. roseus* Var. A.

#### SUMMARY AND CONCLUSIONS.

The 54 strains of the cocci in the American Museum collection group themselves very definitely according to pigment production and other characters into five distinct classes. The correlation of the various morphological and biochemical properties bears out the work by the Winslows and justifies their recognition of five genera among the Coccaceae outside of the diplococci and streptococci.

Six of the strains give faint growth on agar, produce no pigment, ferment sugars very actively and belong, therefore, to the genera *Diplococcus* and *Streptococcus*. Twelve strains produce white pigment, good growth, moderate acidity, are generally gram-positive, liquefy gelatin slowly, if at all, and conform in every way with the genus *Albococcus*. There are three exceptions in this group, which produce no acidity in lactose and saccharose and probably form the connecting link between the true albococci and micrococci.

Orange-pigment producers number 15. This group is distinguished from the white by meager growth, reduction of nitrates, and rapid liquefaction of gelatin. It agrees perfectly with the genus *Aurococcus*. Three strains are packet formers and in other prop-

TABLE 9.  
TABULAR KEY TO THE GENERA AND SPECIES OF COCCACEAE

GENUS	SPECIES	VARI- ETY	SOURCE	CELL GROUPING	DIMENSION AVER- AGE IN $\mu$	GRAM GENERALITY	GROWTH AT		ACID PRODUCTION IN			BREAKING DOWN OF PEPTONE TO $\text{NH}_3$	REDUCTION OF NITRATE TO $\text{NO}_2$	LIQUEFACTION OF GELATIN	CHROMO- GENESIS
							20° C.	37° C.	Dext.	Lact.	Sacch.				
Paracoccaceae	Str. Gracilis.....	....	Parasitic	No packets	0.8	—	Faint	Same	+	+	+	+	—	+	None
	Alb. Rheumaticus.....	....	"	"	0.8	—	Faint	Same	+	+	+	+	—	+	None
	Pyogenes.....	....	"	"	0.8	—	Meager	Better	+	+	+	+	—	+	White
	Uvae.....	....	"	"	0.6	—	Good	Same	+	+	+	+	—	+	White
	Tetragenus.....	....	"	Tetrads	0.6	—	Good	Better	+	+	+	+	—	+	White
	Candidus.....	....	"	No packets	0.6-0.7	—	Good	Same	+	+	+	+	—	+	White
	Epidermidis.....	....	"	"	0.6-0.7	—	Good	Same	+	+	+	+	—	+	White
	Epidermidis.....	....	"	"	0.9	—	Good	Same	+	+	+	+	—	+	White
	Candicans.....	A	"	"	0.7	—	Good	Same	+	+	+	+	—	+	White
	Aureus.....	....	"	"	0.7	—	Good	Same	+	+	+	+	—	+	White
Aur.	Aurantiacus.....	....	"	"	0.7	—	Meager	Better	+	+	+	+	—	+	Orange
	Mollis.....	....	"	"	0.7	—	Meager	Better	+	+	+	+	—	+	Orange
	Mollis.....	A	"	"	0.7	—	Meager	Better	+	+	+	+	—	+	Orange
	Citrus.....	....	Saprophytic	"	0.6	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
M.	Citrus.....	....	"	"	0.6	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Citrus.....	A	"	"	0.8	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Flavus.....	....	"	"	0.8	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Luteus.....	....	"	"	0.7	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
S.	Lutea.....	....	"	"	0.7	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Flava.....	....	"	Packets	0.7	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Flava.....	A	"	"	0.7	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Flava.....	B	"	"	0.7	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
R.	Citrea.....	....	"	"	0.8	—	Abundant	Poorer	+	+	+	+	—	+	Yellow
	Aurantiaea.....	....	"	"	0.8	—	Abundant	Poorer	+	+	+	+	—	+	Orange
	Rosens.....	....	"	Irregular groups	0.8	—	Good	Poorer	+	+	+	+	—	+	Red
	Rosens.....	A	"	"	0.8	—	Good	Poorer	+	+	+	+	—	+	Red
	Fulvus.....	....	"	"	0.8	—	Abundant	.....	+	+	+	+	—	+	Red

erties resemble the sarcinae and are therefore classed with that group, in spite of their formation of orange pigment.

The yellow-pigment group is represented by 15 strains which present a very distinct and definite picture. In its abundant growth, generally gram-negative reaction, and lack of fermentative power, this group corresponds to the genus *Micrococcus*. Seven of the strains which form packets are classed with the three orange packet formers under the genus *Sarcina*.

Five strains produce a red pigment. These, in abundant growth, gram-negative reaction, reduction of nitrates, and lack of fermentative and liquefying power, form a distinct group defined by the Winslows as the genus *Rhodococcus*.

Two new biochemical tests have been applied in this study. The value of saccharose as a differentiating test among the cocci is doubtful. The ammonification of peptone promises, however, to be a valuable additional test both for generic and specific differentiation. This reaction merits further study.

The broad generic outline as laid down by the Winslows is shown to be valid and well established. Their specific types are, however, apparently too broad and inclusive and further study may bring to light new type centers. My work justifies the recognition of *Alb. ureae* and *S. aurantiaca* as distinct species and points to the probable existence of several others. More exhaustive study along this line is highly desirable.

A summary key to the genera and species of Coccaceae is given in Table 9, p. 451.

## THE MORPHOLOGY OF SPOROTHRIX SCHENCKII IN TISSUES AND IN ARTIFICIAL MEDIA.\*

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A point of some interest in connection with the study of fungi of various kinds is the difference that appears in the morphology of the organisms when growing in the living tissues and when growing on artificial media. The variations, for example, of actinomyces and blastomyces in this respect are well-known phenomena. Similar observations on sporothrices have also been observed and it is the purpose of this paper to inquire further into this matter with a view to determining under what conditions such variations in morphology occur.

In living tissues sporothrix organisms always appear as elongated or oval bodies fairly uniform in size and often showing distinct budding processes (Fig. 1). The arrangement is usually single, but frequently two or more are found end to end, and at times several may be arranged radially about a central spore. In experimental sporotrichosis as a rule these forms are numerous in the tissues and easily stained. In the human body they may at times be present in large numbers but usually they are so few in number that it is difficult or impossible to find them. For convenience I shall call these forms, "tissue forms."

Branched mycelial filaments are not found in the living tissues either in the human or the experimental disease. These mycelial threads or hyphae develop abundantly on practically all the artificial media that have been used and at their sides and extremities form numerous spores, oval in shape and differing decidedly in appearance from the tissue forms. In addition to the filaments and spores, typical tissue forms may also be seen in small numbers in artificial culture, especially in fluid media, and may present budding processes like those in tissues.

Let us first compare the method of germination of mycelium

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from spores, with the process of budding as observed in the tissue forms. In the former a bulging may be seen on one side of the spore looking at first not unlike a pseudopod. This increases and either extends in one direction, in which case the original spore will appear at the extremity of the filament or, as often happens, the pseudopod-like structure immediately divides into two branches proceeding in opposite directions, in which case the original spore appears at the side of the filament. Not infrequently, too, the filaments may arise at opposite sides of the spore or several may thus arise. The formation of buds in tissue forms, on the other hand, occurs as a very minute protrusion of protoplasm

from the end of the organism and never from the side. The daughter cell at first is very small but soon increases in size, the pedicle remaining very fine and delicate. This process is quite comparable to the formation of spores at the ends and along the sides of the filaments, but it is evidently quite different from the formation of mycelial threads. The factors which control the formation of the one or the other apparently

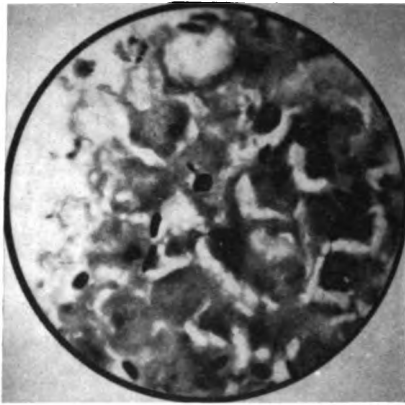


FIG. 1.—Spindle forms of sporothrix in pus from an infected mouse. Note the budding processes.  $\times 1,200$ .

have not been investigated, nor do we know the conditions of their development.

In order to investigate this point the cultivation of sporothrices in various media and under various conditions was made. It was found that when the sporothrix is cultivated in certain body fluids, the tendency to form branching filaments is markedly diminished and in some instances quite absent, whereas in general the tendency to develop tissue forms is decidedly increased. In urine, joint fluid, cerebrospinal fluid, and ascites fluid, the sporothrix thrives, forming a growth which after a few days settles to the bottom of the tube, the fluid above remaining quite clear. In all the above

media not only are branching filaments with many spores seen, but also some tissue forms with budding processes may be found. When the sporothrix is grown in defibrinated blood, however, the number of tissue forms as a rule greatly predominates, in some tubes scarcely a filament occurring. In washed blood and in serum the same is true, though possibly not to the same extent. Heated and unheated sheep serum, human serum, and dog serum all give about the same result, there being no constant or appreciable differences. Distinct growth takes place in all with the occurrence of many typical tissue forms, the filaments being infrequent. In defibrinated blood after growth of about two weeks it is common to see groups of the budding tissue forms arranged radially in large clusters of 20 to 30 or even 50 or more organisms around a central spore or very short filament and simulating, in a measure, actinomyces (Fig. 2).

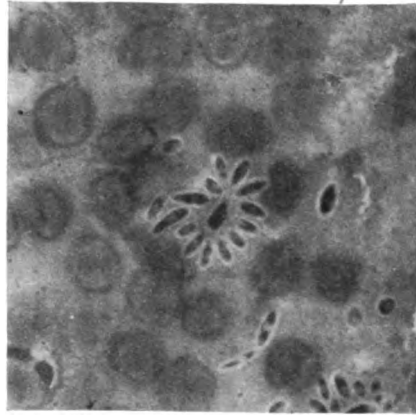


FIG. 2.—Sporothrix growing in pure blood *in vitro*. Note the spindle forms arranged in ray-like clusters.  $\times 1,200$ .

Growth of sporothrix in the serum and blood of rabbits which were immunized against the same organism and whose serum readily agglutinated various strains of sporothrix,<sup>1</sup> did not appear as abundant as in normal fluids. However, some growth did occur which in microscopic appearance was not essentially different from that occurring in normal fluids, there being both tissue forms and some filaments present.

When cultures in blood are grown under paraffin oil, similar or even better results are obtained, there being usually little else than tissue forms present. This is also true, but to a less extent, of broth cultures when grown under oil, a fact which would suggest that the partial anaerobic conditions play a rôle in determining

<sup>1</sup> *Jour. Infect. Dis.* 1913, 12, p. 140.



the morphology of the organism. The sporothrix is strictly aerobic. If a very small amount of oxygen is permitted to enter a tube of inoculated glucose agar, proliferation occurs with the formation of some hyphae but also with the production of a large number of the elongated forms quite like the tissue forms. It may be pointed out here that a somewhat similar observation has been made by Hamburger<sup>1</sup> in connection with blastomyces, in which case certain involution forms appear in anaerobic, glucose agar cultures after several days.

Further observations were made *in vitro* on the growth of sporothrix when in contact with sterile animal tissues. Such

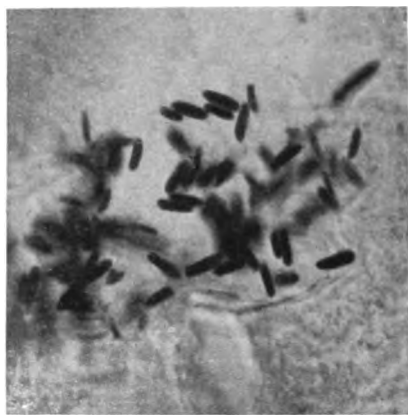


FIG. 3.—Spindle forms of sporothrix growing in sterile kidney tissue *in vitro*.  $\times 1,200$ .

tissues including spleen, liver, and kidney, when placed in test tubes and partially immersed in serum, form an excellent medium for this organism, the growth after a few days covering the surface as a grayish soft mat. Smears from the surface show both mycelial branching forms and tissue forms. On examining stained sections of these tissues after 10 days or two weeks one sees that the sporothrix has already invaded the

tissues to a considerable extent. Directly on the surface may be seen some mycelia but in the dead tissues beneath, there occur in enormous numbers, only the typical elongated tissue forms (Fig. 3) such as appear in the human and the experimental disease. Here branching filaments are never seen. It is an interesting fact that in agar cultures, for instance, branching filaments readily penetrate the subjacent agar in all directions, but they are not able to penetrate either living or dead tissues under similar conditions. It therefore appears that, artificially by using blood or dead sterile tissue under aerobic conditions, we can obtain forms of sporothrix practically identical with those occurring in living tissues.

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 201.

Reference may here be made to a strain of sporothrix isolated and described by Splendore and referred to by Gougerot<sup>1</sup> as *Sporothrix asteroides*. This organism is characterized by the formation, *in vivo* only, of star-like structures or asteroid forms consisting of a central body with radially arranged, elongated spores. Inasmuch as raylike forms of ordinary sporothrices are commonly seen in tissues, it is not unlikely that in this strain the tendency to form such asteroid clusters is more marked than usual and that they are probably not distinctive structures in themselves.

These facts remind one somewhat of the behavior of the ray forms of actinomyces in tissues. The club forms of this organism which may be roughly compared with, though probably are not analagous to, the "tissue forms" of sporothrix, do not occur on artificial media unless, as has been observed by Wright, body fluids, such as blood serum or pleuritic fluid, are added. Wright regards the club formation as a process designed to protect the organism from the tissue reactions. Others regard the clubs as degeneration forms and still others as organs of fructification. Whatever may be their function the similarity in form of sporothrix, when grown in blood, to the ray fungus is at times rather striking, a fact which may be associated with the observation made by Widal and Abrami<sup>2</sup> that a group relationship based upon serum tests exists between sporothrix and actinomyces.

The observations recorded in this paper concern various strains of sporothrix including a strain isolated in this country by Hektoen in 1898 and named by him *Sporothrix Schenckii*, and also a strain obtained from Dr. Gougerot of Paris and there known as *Sporothrix Beurmanni*. It may be stated that no essential differences in behavior were noted between the various strains when grown in the different media and under the conditions as detailed above.

#### SUMMARY.

In human and experimental sporotrichosis the sporothrix always appears as oval or elongated bodies and never forms distinct hyphae.

<sup>1</sup> Kolle and Wassermann, *Handbuch der Path. Microorg.*, Zweite Auflage, Bd. V, p. 225.

<sup>2</sup> *Ann. de l'Inst. Pasteur*, 1910, 24, p. 1.

On ordinary artificial culture media, long branching hyphae appear together with some morphological forms like those found in tissues.

Sporothrix grows readily in various body fluids. In some of these fluids, especially blood, the tissue forms may greatly predominate.

Sterile animal tissues *in vitro* furnish an excellent medium for growing sporothrix and here the tissue forms appear exclusively.

Partial anaerobic conditions seem to play a rôle in causing these morphological variations though there are probably other determining factors.

## THE MEIOSTAGMIN AND EPIPHANIN REACTIONS IN THE DIAGNOSIS OF CARCINOMA.\*

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In 1910 Ascoli and Izar<sup>1</sup> first employed the stalagmometer in the diagnosis of malignant tumors, and in 1912 Rosenthal<sup>2</sup> applied the epiphanin reaction to the differentiation of carcinoma proteins from normal organ proteins and suggested its application in the diagnosis of carcinoma.

Previous to the appearance of Rosenthal's work, I had made attempts to adapt the epiphanin reaction to the diagnosis of carcinoma. In none of the preliminary experiments, however, were results obtained which were beyond the range of error as established by the controls. Subsequent to Rosenthal's report, a comparative study of the meiostagmin and epiphanin reactions in a limited series of cases was undertaken. This series consists of 20 cases, in all of which the clinical diagnosis was established without any doubt.

### THE MEIOSTAGMIN REACTION.

To obtain a reliable antigen, proved rather difficult. Micheli and Catoretti,<sup>3</sup> and Verson<sup>4</sup> used antigens from pancreas and thyroid tissue. I prepared antigens from human carcinoma, the thyroid gland (both exophthalmic and adenomatous), placenta (washed free from blood), and dog pancreas. Preliminary titrations demonstrated the pancreas antigens to be far more active than those obtained from thyroid, placenta, or even carcinoma tissue, and in the subsequent tests this was the antigen employed. The antigen was prepared by desiccating the tissue, which had been previously rubbed up in a mortar with quartz sand, in a current of air at about 37° C. The powdered desiccate was then treated with methyl alcohol, in the proportion of 1-4, at 50° C. for 24

\* Received for publication February 14, 1913.

<sup>1</sup> *München. med. Wchnschr.*, 1910, 57, p. 403.

<sup>2</sup> *München. med. Wchnschr.*, 1910, 57, p. 1122.

<sup>3</sup> *Ztschr. f. Immunitätsf.*, 1912, 15, p. 37.

<sup>4</sup> *Wien. klin. Wchnschr.*, 1910, 23, p. 1102.

hours with frequent shakings. This was then filtered while still hot through a Schleicher and Schuell filter No. 590. After cooling, the filtrate was filtered again. This extract was then titrated in water emulsions of various dilutions with normal sera until a dilution was obtained that would not cause an increase of more than one drop as determined by a Traube's stalagmometer. All sera were 24 hours old. The technic as revised by Ascoli and Izar<sup>1</sup> was followed. Two control sera, one noncarcinomatous and one carcinomatous, were tested with each serum. The reactions were completed by heating the serum and antigen mixtures (9-1) in a water bath at 50° C. for one hour. The graduations on the stalagmometer were such that one drop of the serum dilutions in general averaged 13 divisions of the scale. Ten of the 11 cases of malignant tumors in this series were carcinomas; one was a multiple myeloma. In all of these, the surface tension of the serum was decreased by the action of the antigen. In all but two, the decrease caused an increase of more than one drop. In only 5 of the sera was the increase greater than 2 drops.

Ascoli originally pronounced only such reactions positive as presented an increase of two drops or more. Later investigators have considered an increase of less than two drops as positive. Koehler and Luger,<sup>2</sup> working with an acetone lecithin extract as antigen, include an increase as low as 12/13 drop as positive. The tendency to assume as positive an increase of less than two drops or even than one drop, undoubtedly accounts for the varying degrees of success attained by different workers with this reaction. According to Ascoli's standard, only 45 per cent of the tumor cases of my series presented a positive reaction, while 11 per cent of the surely noncarcinomatous cases reacted positively. Were these figures to be revised according to the standard of Koehler and Luger, however, 100 per cent of the tumor cases and 33 per cent of the other cases give a positive reaction. During the course of these experiments, it was accidentally discovered that a very slight trace of soap was capable of decreasing surface tension as determined by the stalagmometer. This also has been noted by

<sup>1</sup> *München. med. Wchnschr.*, 1910, 57, p. 1170, 2129.

<sup>2</sup> *Wien. klin. Wchnschr.*, 1912, 25, p. 1114.

Kelling.<sup>1</sup> Not infrequently the water-antiserum controls presented a slight decrease in surface tension which I was unable to explain. In this series it is interesting to note that the reactions in two cases of myelogenous leukemia were negative, while that of a case of multiple myeloma (myelocytoma) was positive.

#### THE EPIPHANIN REACTION.

According to Weichardt,<sup>2</sup> the epiphanin reaction depends in part on an acceleration of the rate of diffusion in a solution, when antigen and its specific antibody are introduced. For demonstration a barium-hydrate-sulphuric-acid system is employed. In this system the sulphuric acid is of such concentration as to exactly neutralize an equal volume of saturated barium hydrate solution. Phenolphthaline in combination with a catalytic agent, strontium chlorid, is used as the indicator. Colloidal substances are said to alter the surface tension of the suspension of finely divided barium sulfate particles so as to increase the absorption of H ions. In this way the point of neutralization is shifted accordingly as the amount of absorption is large or small. A definite quantity of the barium hydrate and sulphuric acid is added to definite amounts of antigen and antibody which have previously been allowed to act on each other; the control is obtained by adding the same quantity of barium hydrate and sulphuric acid to the same amounts of antigen and antibody, but before there has been time for a reaction between antigen and antibody to take place. The degree to which the point of neutralization has been shifted in both instances is then determined by titrating with N/1000 H<sub>2</sub>SO<sub>4</sub> and the difference between the two determined.

In this manner the variations for three different serum dilutions (10<sup>4</sup>, 10<sup>6</sup>, 10<sup>8</sup>), have been determined and plotted in the form of a curve. Figures on the axis of ordinates represent cubic centimeters of N/1000 H<sub>2</sub>SO<sub>4</sub>; figures on the abscissae represent the different serum dilutions (10<sup>2</sup>=1-100, 10<sup>4</sup>=1-10000, etc.). An attempt was made to use the same antigens in the epiphanin reactions as were employed in the meiostagmin reaction. This was done in 5 of the 20 sera which were tested. In the remaining 15

<sup>1</sup> *Arch. f. Verdauungskr.*, 1912, 18, p. 164.

<sup>2</sup> *Ztschr. f. Immunitätsf.*, 1910, 4, p. 651.



antigen and antiserum of beaker 1 have had an opportunity to act one on the other before the addition of the chemical system.

A series of blind titrations in which distilled water was substituted for both antigen and antiserum was first made. This series consisted of several hundred titrations to determine the range of error in this reaction. Charts 1 and 2 represent some of the

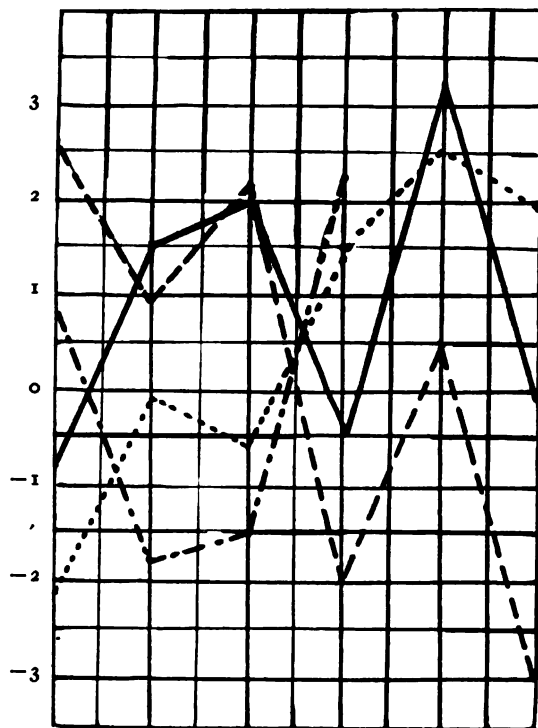


CHART 2.—Epiphanin reaction. Titrations with reagents only. Four curves obtained by four-glass method.

extremes of the range of error in these titrations. During the course of the work von Angerer and Stoetter<sup>1</sup> published a single-glass method by which they are able to decrease their range of error very materially. That this should be possible is readily understood. The four-glass method requires just four times the amount of reagents, serum, and antigen, and four times as much

<sup>1</sup> *München. med. Wchnschr.*, 1912, 50, p. 2035.



manipulation as the single-glass method. The single-glass method employs but one beaker. Its contents and the order of their addition are the same as those of beaker 1 of the four-glass method. It is assumed by von Angerer and Stoetter that the neutral point

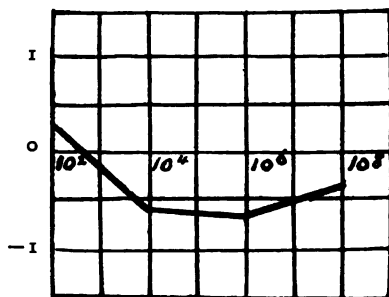


CHART 3.—Epiphanin reaction.  
Normal serum.

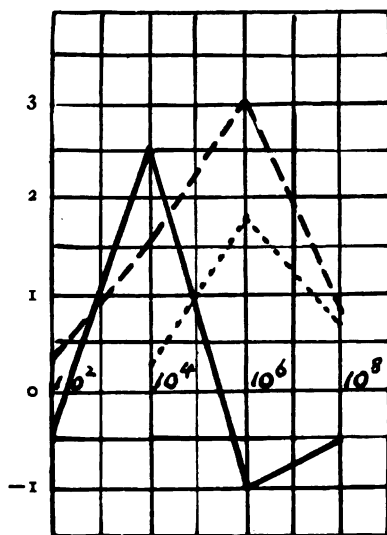


CHART 4.—Epiphanin reaction. Serum  
from carcinoma of neck.

Figures on the abscissae represent different dilutions of serum,  $10^2=1-100$ ,  $10^4=1-1000$ , etc. Reactions obtained with Antigen I, a glycerin-water extract of a mammary carcinoma. Four-glass method.

Meiostagmin reactions with same serum and Antigen II, an alcoholic extract of pancreas.

Before heating	$49^{+1}$ drops
After " "	$49^{+8}$ "
H <sub>2</sub> O control	$49^{+2}$ "

( $49^{+1}$  gtt. = 49 drops and approximately  $1/13$  of a drop.  
 $49^{+8}$  gtt. = 49 drops and approximately  $8/13$  of a drop, etc.)

— Antigen III (Alcoholic extract of placenta).  
 ..... Antigen IV (Alcoholic extract of thyroid).  
 - - - - - Antigen II.

Four-glass method.

Meiostagmin reaction with same sera.

Antigen	Before heating	After heating	Control
III	$50^{-8}$ drops	$50^{-1}$ drops	$50^{-2}$ drops
IV	$50^{-8}$ "	$50^{-4}$ "	$50^{-2}$ "
II	$50^{-8}$ "	53 "	$50^{-2}$ "

of the combined contents of beakers 2 and 3 is the same as that for the barium-hydrate-sulphuric-acid system; they assume the latter to be a constant, i.e., 0.

In a series of several hundred blind titrations with the single-glass method, my range of error was but little if any greater than

———— Mammary carcinoma, two years after operation, recurrence in scar, bone metastases, no cachexia; antigen II; four-glass method.

Antigen	Before heating	After heating	Control
II	50 <sup>-6</sup> drops	54 <sup>-3</sup> drops	50 <sup>-5</sup> drops
III	50 <sup>-6</sup> "	50 <sup>-1</sup> "	50 <sup>-5</sup> "
IV	50 <sup>-6</sup> "	50 <sup>+1</sup> "	50 <sup>-5</sup> "

----- Mammary carcinoma, first noticed 7 months before, age 35 years; no cachexia; antigen II; four-glass method.

Meiostagmin reaction for same serum: antigen II, before heating, 49<sup>-1</sup> drops, after heating 50<sup>-2</sup> drops, control, 49<sup>-1</sup> drops.

..... Mammary carcinoma, 3 years after operation, carcinoma of axillary glands at this time, no cachexia; antigen II; four-glass method.

Meiostagmin reaction for same serum: antigen II, before heating, 49<sup>-2</sup> drops, after heating 51<sup>-4</sup> drops, control, 49 drops.

----- Mammary carcinoma, entire right breast and pectoral muscles involved, 67 years, marked cachexia; antigen V, glycerin water extract; of cervix carcinoma; four-glass method.

Meiostagmin reaction for same serum:

Antigen	Before heating	After heating	Control
II	51 <sup>-9</sup> drops	52 <sup>-4</sup> drops	51 <sup>-7</sup> drops
VI	51 <sup>-9</sup> "	51 "	51 <sup>-7</sup> "

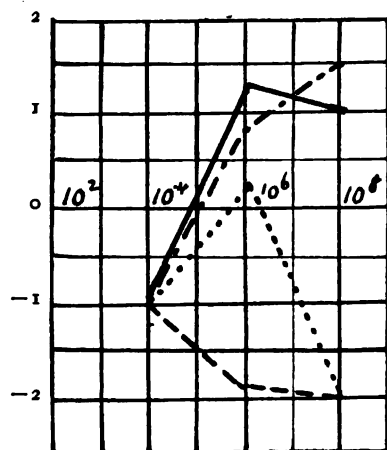


CHART 6.—Epiphantin reactions.

age 18 years, well nourished; antigen I; four-glass method.

Meiostagmin reaction for same serum: antigen II, before heating 49<sup>-2</sup> drops, after heating 50<sup>-5</sup> drops, control, 50<sup>-8</sup> drops.

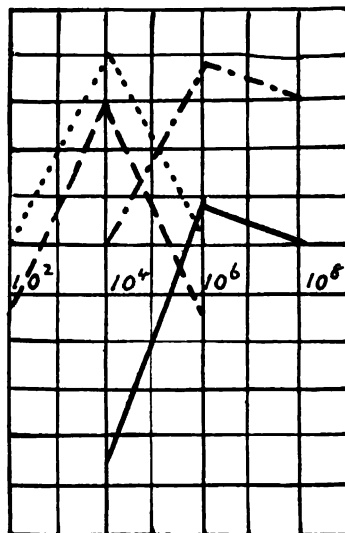


CHART 5.—Epiphantin reaction. Four cases of carcinoma.

———— Carcinoma of oesophagus, first symptoms of stenosis 1 year ago, age 65, loss of weight 152 lbs., former weight 240 lbs.; antigen I; four-glass method.

Meiostagmin reaction for same serum: antigen II, before heating 50<sup>-6</sup> drops, after heating 52<sup>-5</sup> drops, control, 50<sup>-1</sup> drops.

----- Case of chronic sciatica, age 20 years, well nourished; antigen I; four-glass method.

Meiostagmin reaction for same serum: antigen II, before heating 51 drops, after heating 52<sup>-6</sup> drops, H<sub>2</sub>O control, 51<sup>-4</sup> drops.

..... Herniotomy, uncomplicated, 5 days after operation, ether anaesthesia, well nourished; antigen V; four-glass method.

Meiostagmin reaction for same serum: antigen II, before heating 52<sup>-9</sup> drops, after heating 52<sup>-4</sup> drops, control, 52<sup>-5</sup> drops.

----- Infected finger, axillary adenitis,

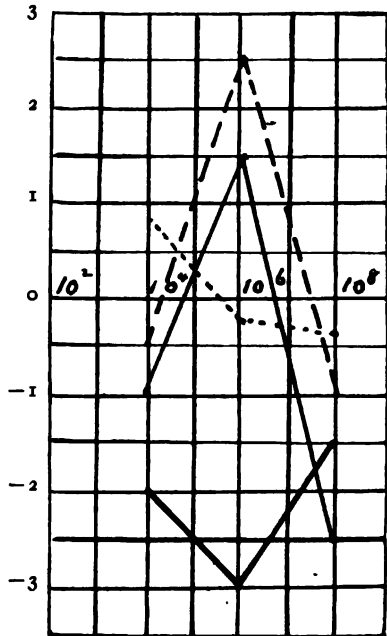


CHART 7.—Epiphanin reactions. Carcinoma of oesophagus, first symptoms of stenosis 4 months ago, loss of weight 104 lbs. in 4 months, present weight 180 lbs.

- Simultaneous titrations, antigen I, four-glass method.  
 - - - - - Antigen I plus antikenotoxin, four-glass method.  
 . . . . . Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating,  $48^{-4}$  drops, after heating  $49^{-3}$  drops, control,  $48^{-1}$  drops.

— Simultaneous titrations; antigen I; four-glass method.

Meiostagmin reaction, same serum: antigen II, before heating,  $48^{-2}$  drops, after heating,  $53^{-2}$  drops, control,  $48^{-4}$  drops.

- - - - - Same serum, antigen I plus antikenotoxin, four-glass method.

. . . . . Same serum, antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating  $48^{-2}$  drops, after heating,  $53^{-2}$  drops, control  $48^{-4}$  drops.

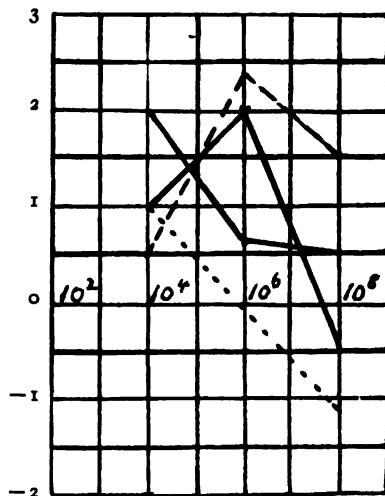


CHART 8.—Epiphanin reactions. Carcinoma of stomach, marked cachexia.

- Simultaneous titrations, antigen I, four-glass method.  
 - - - - - Antigen I plus antikenotoxin, four-glass method.  
 . . . . . Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating,  $48^{-4}$  drops, after heating,  $49^{-3}$  drops, control,  $48^{-2}$  drops.

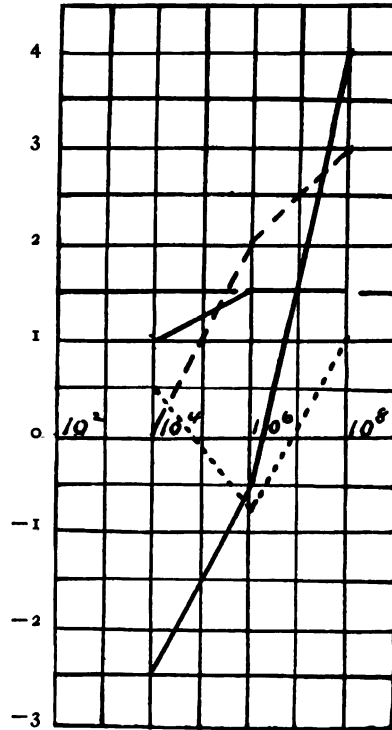


CHART 9.—Epiphanin reactions. Carcinoma of stomach, marked cachexia.

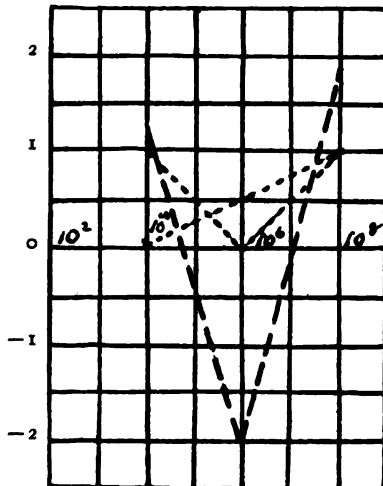


CHART 10.—Epiphanin reactions. Carcinoma of stomach, slight cachexia.

- . . . . . Two curves, simultaneous titrations, antigen I, single-glass method.  
 - - - - - Antigen I plus antikenotoxin, four-glass method.

Meiostagmin reaction of same serum: antigen II, before heating  $48^{-4}$  drops, after heating,  $49^{-6}$  drops, control,  $48^{-12}$  drops.

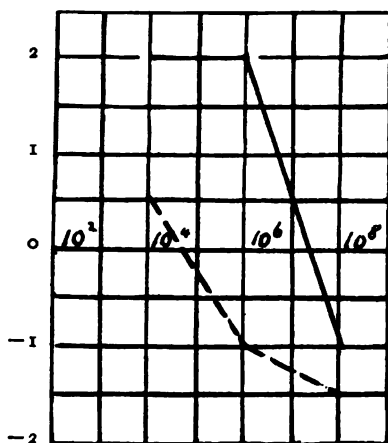


CHART 11.—Epiphanin reactions. Pulmonary tuberculosis, exudative pleuritis, slight cachexia.

— Antigen I, four-glass method.  
 - - - Same serum, antigen I plus antikenotoxin, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating,  $49^{-1}$  drops, after heating,  $50^{-6}$  drops, control,  $49^{-3}$  drops.

— Simultaneous titrations, antigen I, four-glass method.  
 - - - Antigen I plus antikenotoxin, four-glass method.  
 . . . . . Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating,  $48^{-8}$  drops, after heating,  $52^{-6}$  drops, control,  $48^{-8}$  drops.

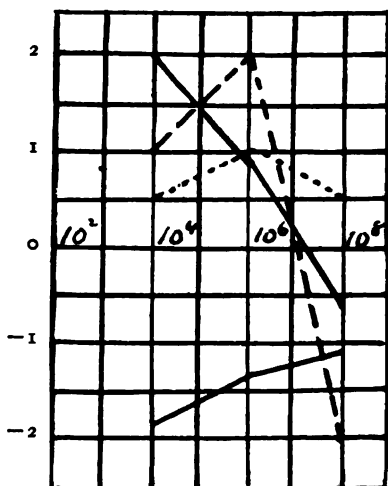


CHART 12.—Epiphanin reactions. Typhoid, 5th week, relapse.

- Simultaneous titrations, antigen I, four-glass method.  
 - - - - - Antigen I plus antikenotoxin, four-glass method.  
 . . . . . Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating, 48 drops, after heating, 49<sup>-4</sup> drops, control, 48<sup>-2</sup> drops.

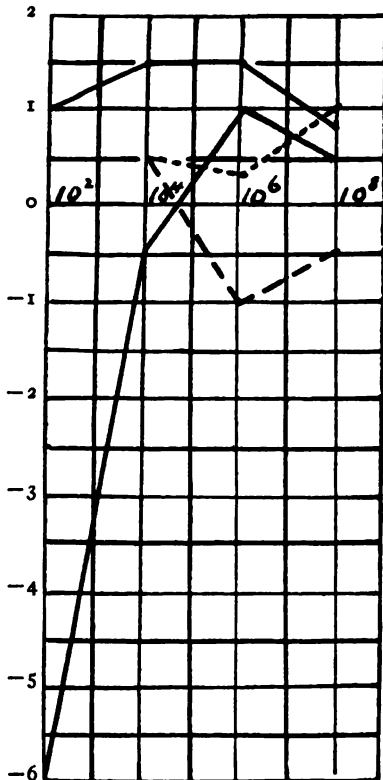


CHART 14.—Epiphanin reactions. Multiple myeloma of clavicle, ribs, vertebrae, pelvic bones, long bones, and skull, extreme cachexia.

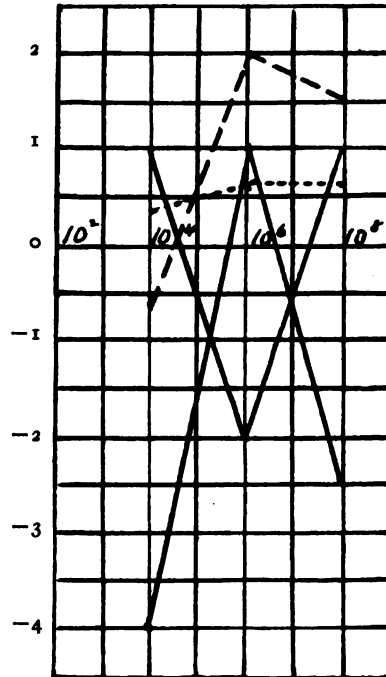


CHART 13.—Epiphanin reactions. Chronic nephritis, uremia.

- Simultaneous titrations, antigen I, four-glass method.  
 - - - - - Antigen I plus antikenotoxin, four-glass method.  
 . . . . . Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating, 48<sup>-3</sup> drops, after heating, 50<sup>-2</sup> drops, control, 49<sup>-9</sup> drops.

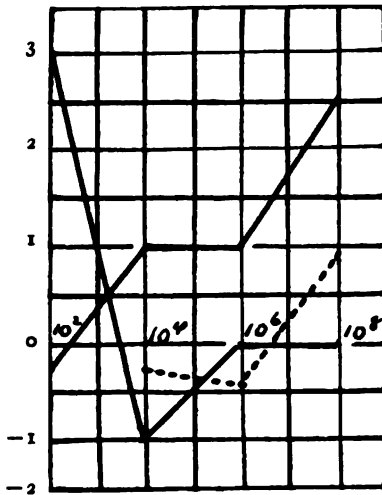


CHART 15.—Epiphanin reaction. Myelogenous leukemia, roentgen ray burn.

— Simultaneous titrations, antigen I, four-glass method.

- - - - - Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating  $49^{-9}$  drops, after heating,  $50^{-4}$  drops, control,  $49^{-8}$  drops.

— Simultaneous titrations, two curves, antigen I, four-glass method.

- - - - - Antigen I plus antikenotoxin, four-glass method.

..... Antigen I, single-glass method.

Meiostagmin reaction of same serum: antigen II, before heating  $47^{-7}$  drops, after heating,  $47^{+1}$  drops, control,  $47^{-4}$  drops.

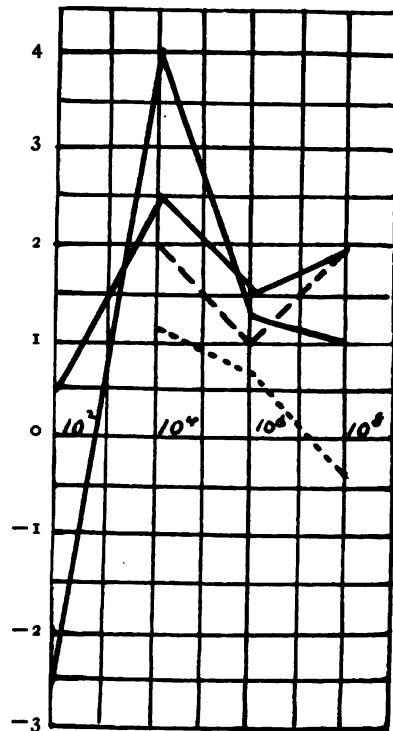


CHART 16.—Epiphanin reaction. Myelogenous leukemia, roentgen ray therapy.

that of von Angerer and Stoetter. Their titrations were made with a barium hydrate solution much more dilute than that employed in the epiphanin reaction as outlined by Weichardt. The avidity of their reagent for CO<sub>2</sub> (the CO<sub>2</sub> of the air is the one great source of error in the epiphanin reaction) must therefore have been a great many times less intense than that of the concentrated reagent ordinarily employed in the reaction. Unfortunately, however, it will be seen that as the range of error decreased with this modified technic so also did the curves plotted from the serum reactions recede, with very few exceptions remaining within the previously established range of error for the single-glass method. In this respect these results resemble those of Korff-Peterson and Brinkmann.<sup>1</sup> The range of error at no time was as great as that obtained by these workers. This in all probability is due to the fact that they may have worked with the mikra-pipette. In the latter half of my series, both the single- and four-glass methods were employed. Double titrations with the four-glass method were made simultaneously for this portion of the series. In no single instance, however, were two like curves obtained by the same method for the same serum and antigen mixtures. The addition of Weichardt's antikenotoxin<sup>2</sup> to my antigens did not produce reactions any more marked than those obtained with plain antigens. All sera used in this reaction were 48-hour sera. From the study of these titrations, Rosenthal's results like those of this series would also appear to be within the range of error.

#### CONCLUSIONS.

A decidedly negative meiostagmin reaction is of more value than a positive one and may be considered of some weight in ruling out carcinoma. A moderately or even strongly positive reaction is not necessarily indicative of malignant tumor.

The epiphanin reaction is valueless in the diagnosis of malignant tumors. The range of error determined by the blind titrations in a measure also explains the results obtained by other workers who have employed this reaction in the diagnosis of diseases other than carcinoma.

<sup>1</sup> *Ztschr. f. Hyg. u. Infektionskrankh.*, 1912, 72, p. 343.

<sup>2</sup> *Ztschr. f. Immunitätsf.*, 1912, 13, p. 383.



## THE PROPERTIES AND AGGLUTINATIONS OF SOME NON-PATHOGENIC VIBRIOS.\*

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In routine examinations for cholera many observers have noted the occurrence of certain organisms morphologically resembling the cholera vibrio, but differing from it in negative agglutination with an anticholera serum, and in the slight or non-pathogenicity to animals. They are usually found in individuals free from any symptoms of disease, and do not give rise to any known lesion in man. These aberrant types are known as the non-cholera vibrios, and their importance rests not so much upon any intrinsic properties of their own as in the fact of their association with the cholera organism in outbreaks of that disease.

During the routine examination carried out at Quarantine, N.Y., in 1911, a great number (over 100) of the non-cholera vibrios were found. With one exception those were all obtained from individuals in good health, the exception being one case in which mild choleraic symptoms were present, but the cholera vibrio was not isolated at the same time. A somewhat suggestive incident noted was that the passengers from one ship, among whom two cholera carriers were detected, also provided the greatest number of non-cholera vibrios isolated at one time.

A certain significance attaches to this event from the point of view both of some relationship between the cholera and the non-cholera vibrios, and of a possible predisposing action upon the intestinal mucosa, the latter a factor which might influence the development of an attack by the cholera vibrio. There is, however, no direct evidence of any such predisposing action at our disposal.

The presence of this type of vibrio in persons from infected ports and from ships upon which cholera carriers were found was in itself a reason to regard them with peculiar interest.

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## MORPHOLOGY.

In describing the morphology of the non-cholera forms isolated at Quarantine, it may be said that the greater number resembled the cholera organism so closely that a separate description of them would be difficult, yet others presented slight or striking peculiarities, which were of interest as negative evidence upon a suspected organism.

For comparison the cholera organism may be described as crescent shaped, broader at the center, and with blunt ends, and from 1.5-2.  $\mu$  in length. The non-cholera vibrios often showed many departures from this type. These differences were frequently of size, and particularly noticeable were long forms, up to 8  $\mu$  in length with blunt ends, and short forms of extreme curvature with pointed ends. These atypical forms in size and shape are so characteristic of the non-pathogenic vibrios that their occurrence in a smear from a fresh culture may well give grounds for the belief that the observer is not dealing with the organism of cholera. At the same time it must not be forgotten that a number of the non-pathogenic vibrios resemble the cholera organism so closely that no difference can be discerned in microscopical examination. The cholera vibrio is very liable to heteromorphism when grown upon new or unsuitable media. Since the new forms thus produced are not of a permanent character, however, replanting upon suitable media will transform them again into the usual shape. These atypical non-cholera vibrios do not show any tendency to conversion to the cholera shape when grown on ordinary media.

## MOTILITY.

Koch, in his original account of the cholera vibrio, emphasized its characteristic motility, and indeed in no other organism but these with the terminal flagella is seen the extraordinary lightning-like darting to and fro as in this type of spirillum. The non-pathogenic vibrios isolated at Quarantine were found to be monoflagellates, and their motility was typically cholera-like.

## CULTURAL CHARACTERS.

Culturally they resemble the cholera vibrio very closely. In alkaline broth and peptone, clouding occurs through the medium with

more or less pellicle formation. In some the presence of floccules is observable. In gelatin stabs, liquefaction occurred with all, forming bubble or stocking-shaped liquefaction. The rapidity varied with each strain, this being frequently faster than that of the cholera vibrio.

On blood agar, hemolytic power was present to a variable extent with the cultures tested, differing in this way markedly from the cholera vibrio.

The fermentation of sugars was in the main similar to that of the cholera vibrio. Saccharose, glucose, and maltose were fermented freely, while lactose was not so generally acted upon (30 per cent).

The indol reaction was not present to any extent, although some gave a delayed color. In no instance was a typical cholera-red obtained with any non-cholera vibrio isolated at Quarantine.

On alkalin agar plates the colonies of the non-cholera vibrios show certain differences from those of cholera. As a rule they appear more globular, and not so flat, are more granular in substance, and with more coarse granules in the centers. The cholera colony is moist and when touched with a platinum needle tends to flow. The non-cholera colony, on the other hand, has not so much this appearance of fluidity, and tends to roll up when touched with the needle. The fecal odor so generally found in cholera cultures is not so pronounced in those of the non-cholera type. In describing the colonies of the non-cholera vibrios, a hard-and-fast line dividing them from the cholera organism cannot be drawn. Some may differ in one or more, or all of the specific characters described above.

Besides this cultural similarity to the organisms of cholera, there were also some striking special properties. Three vibrio strains showed considerable pigment formation and another was a gas former. The latter was of interest, gas formation not being a characteristic of any other member of the cholera-like group isolated at Quarantine.

#### PATHOGENESIS.

The pathogenicity to animals of the non-cholera vibrios isolated during various epidemics has differed widely. The pathogenicity

of our non-cholera vibrios was tested by simple feeding and with Pfeiffer's peritoneal inoculation test with living cultures upon guinea-pigs and rabbits. No pathogenic power was demonstrated, even though large doses up to a whole agar culture at one time were given to a 200-gram guinea-pig.

#### AGGLUTINATION.

In the tests for the differential agglutination of the cholera and non-cholera vibrios, a serum of high agglutinating power was used. The cholera vibrio is particularly sensitive to the action of a strong immune serum and will agglutinate in serum dilution up to 1-10,000. The non-cholera vibrios showed a slight tendency to agglutinate with the anticholera serum only at very low dilution, 1-10, and so forth, but none at higher dilutions. A difference was also observed in the reactions with an anticholera human and anticholera horse serum. A good agglutination with a serum dilution of 1-200 was considered a positive reaction for the cholera vibrio. For purposes of differential diagnosis, no matter what cultural or morphological resemblances there were to the cholera vibrio, a negative agglutination with the antiserum absolutely ruled out any vibrio from the cholera class. In earlier times a microscopical finding was considered sufficient evidence upon which to base a positive decision for cholera if the special cultural tests were also positive. The recognition of the existence of the non-cholera vibrios has made such a means of diagnosis of tentative value only, reliance being placed now mainly upon a positive agglutination with an anticholera serum.

The morphological and cultural resemblances between these various vibrio strains suggested the possibility of a relationship between them. It was thought that some light could be thrown upon the question by preparing immune sera from a number of them and testing each vibrio strain with the heterologous agglutinating sera. The preparation of antisera from a great number would have been a long and laborious procedure, so that to bring the attempt within reasonable bounds, from 20 laboratory cultures showing good growth and morphological characters, rabbit agglutinating sera were prepared. A preliminary examination of the normal serum

from each rabbit did not disclose the presence of any agglutinating power upon the vibrio cultures used. The technic employed in the preparation of the immune serum was modified somewhat from the usual method. It was found that four weekly inoculations with three dead cultures and one living one in increasing doses did not produce a serum with any degree of agglutinating power. So that four further intraperitoneal, immunizing doses with living cultures were given at weekly intervals. This yielded a better specific serum, but even this was of low titer. Some degree of agglutination was obtained in dilution of 1-80, a better working strength

TABLE 1.  
AGGLUTINATION TABLE.

Anti- sera	Vibrio Cultures																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.....	+	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-
2.....	+	+	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-
3.....	+	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-
4.....	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-
5.....	-	-	-	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
6.....	-	-	-	-	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-
7.....	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-
8.....	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-
9.....	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
10.....	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
11.....	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+
12.....	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+
13.....	-	-	-	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
14.....	-	-	-	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-
15.....	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
16.....	-	-	-	+	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-
17.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
18.....	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
19.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
20.....	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+

+ = positive agglutination.

- = negative agglutination.

was found to be one of 1-40. This low agglutinating power of the antisera prepared from the non-cholera vibrios may be compared with the powerful agglutinating serum obtained by similar methods from the cholera vibrio. It would seem that the pathogenic organism is capable of producing a greater activation of the tissue cells of the body, and the corresponding response is a production of immune serum of high potency.

The agglutination tests with the immune sera are represented in Table 1, and show a great number of cross-agglutinations with the vibrio cultures. All the antisera but one agglutinated not only their

own homologous cultures, but also cultures of other vibrio strains. The number of interagglutinations is variable for each serum; there may be as many as six or as few as one. A point of interest in these



FIG. 1.—Cholera vibrio from direct smear.  $\times 1000$ .

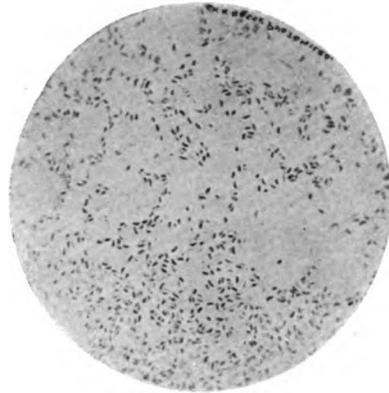


FIG. 2.—Cholera vibrio from 24-hour agar culture (Dr. J. Drennan).  $\times 1000$ .

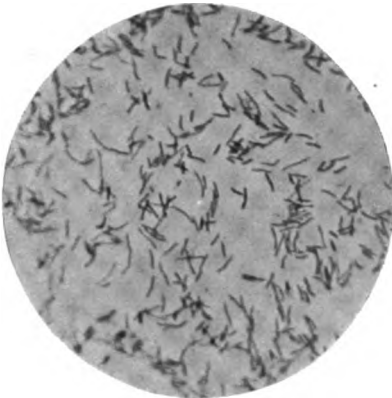


FIG. 3.—Non-cholera vibrio. Long form from 24-hour agar culture.  $\times 1000$ .

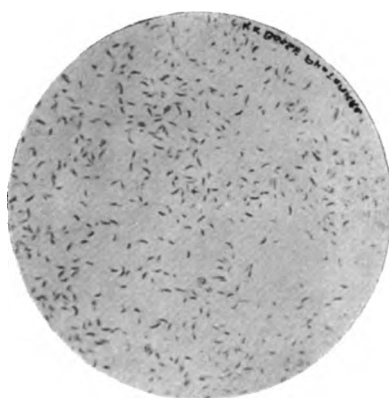


FIG. 4.—Non-cholera vibrio. Short form from 24-hour agar culture.  $\times 1000$ .

reactions is that although the majority are reciprocal in nature, this is not always a rule.

Out of 400 tests represented in Table 1, 75 are positive, made up of 19 homologous and 56 heterologous agglutinations. Thirty-six of the latter were reciprocal.

On careful observation of the table, the presence of certain group agglutinations is indicated by these results, and the various vibrios, by a kind of chain of positive interagglutinations, are seen to arrange themselves into groups, the members of which react only among themselves. Furthermore, the grouping, as indicated above, was accentuated by similar peculiar agglutinative reactions with the anticholera human and horse sera. One vibrio culture did not develop an immune serum of any strength in the animal used, although it was positively agglutinated by other heterologous sera.

It may be justifiable to assume from these results that in the great number of cholera-like non-pathogenic vibrios found we are dealing with the closely allied members of several large groups, and not a great number of separate species of organisms of this nature.

There naturally arises at this time the question of the probable relationship between the non-pathogenic vibrios isolated during cholera epidemics, and the cholera vibrio. From the morphological and cultural characters of some of them, evidences of near relationship could be deduced. Against these we have to place the negative agglutination with the specific immune serum, the non-pathogenicity to animals, and the hemolytic powers.

Some grounds for belief in the distinct individuality of these varieties was furnished by a series of tests with the cholera vibrio and the antisera prepared from the non-cholera cultures, for in every case a negative agglutination reaction was obtained.

As a causative factor of disease in man, the position of the non-cholera vibrios is still in doubt. They have been regarded by some authorities as aberrant or "transitional" forms of the cholera vibrio. Zlatogoroff<sup>1</sup> by his experiments has endeavored to prove that a cholera-like vibrio may acquire agglutinative powers by various methods of cultivation and passage through animals. He maintains that the vibrios found in association with the cholera vibrio may be attenuated varieties, capable at any time of taking on pathogenic powers. McLaughlin and Whitmore,<sup>2</sup> repeating Zlatogoroff's experiments with non-cholera vibrios found in the Philippines,

<sup>1</sup> *Centralbl. f. Bakteriol.*, I, Orig. 1908, 48, p. 684.

<sup>2</sup> *Philippine Jour. Sci.*, 1910, 5, p. 403.

state: "Our vibrios isolated from the human intestine, from water or other sources, which are negative to agglutination with anti-cholera serum, and classified as non-cholera, do not develop agglutinability to cholera serum when treated as directed by Zlatogoroff." The fact remains that the non-cholera vibrios do exist in the human body without the manifestation of any untoward symptoms. A similar rôle is played by the cholera organisms in the case of cholera carriers. The non-cholera organisms are not, however, totally devoid of pathogenic power, for under experimental conditions of lowered vitality, such as were produced by Koch<sup>1</sup> in his experiments upon animals with the non-pathogenic vibrios of Deneke and Miller, it was shown that a rapid and fatal illness with dissemination by the blood and a general septicemia could readily result.

TABLE 2.  
COMPARISON.

	Cholera Vibrio	Non-Cholera Vibrios
Morphology.....	Sickle-shaped crescents, blunt ends 1.5-2 $\mu$	Same as cholera Frequently long forms 8 $\mu$ with blunt ends, and short forms with pointed ends
Motility.....	Characteristic torpedo-like	Cholera-like
Cultural properties	Good growth on alkaline media; fecal odor	Frequently cholera-like (exceptions: pigment, and fecal odor sometimes absent, gas formation)
Pathogenicity.....	Pathogenic to animals	Non-pathogenic to animals
Hemolysis.....	Not hemolytic	Hemolytic powers variable
Sugar fermentation.	Saccharose, etc., fermented	Saccharose, glucose, and maltose fermented; lactose sparingly
Gelatin liquefaction	Liquefies gelatin	Liquefies gelatin at various rates, but usually more rapidly
Indol reaction.....	Constant with pure cultures	Absent or slight
Agglutinations.....	Positive agglutination with anti-cholera serum Negative with non-cholera sera	Negative agglutination with anticholera serum. Heterologous agglutinations with non-cholera sera

#### SUMMARY.

The non-pathogenic vibrios are monoflagellate organisms at times found associated with the cholera vibrio in cholera outbreaks. They resemble morphologically the cholera organism, the exceptions being long forms with blunt ends, and short forms with pointed ends.

*Culturally* there are only slight differences from the cholera vibrio. These are noticeable in the colonies on agar plates, and

<sup>1</sup> *Ztschr. f. Hyg. u. Infektionskrankh.*, 1893, 14, p. 319.



in the absence of the indol reaction. The odor of the cultures is not constantly fecal in character. The action upon sugars is very similar to that of cholera. They have distinct hemolytic powers, and gelatin is readily liquefied. Two distinctive characters were observable. These were pigment formation, and gas in saccharose gelatin media. They are non-pathogenic to animals in even large doses, and show peculiar agglutinative reactions with antisera from other like vibrios, suggesting a measure of relationship between the various vibrio strains.

In carrying out this investigation I am indebted for valuable assistance to Dr. E. C. Baldwin, director of the Quarantine Laboratory.

## STUDY OF AN OUTBREAK OF SEPTIC SORE THROAT OCCURRING IN CONCORD (N.H.), JANUARY, 1912.\*

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Durham, North Carolina.

Epidemics of tonsillitis or "septic sore throat," other than those due to Klebs-Loeffler bacillus of diphtheria, have not until recent years attracted much attention in this country. In May, 1911, there was an epidemic in Boston which was studied by Winslow<sup>1</sup> and the Massachusetts Board of Health.<sup>2</sup> Seven epidemics of sore throat due to milk, all of which occurred in Europe, are reported by Rosenau.<sup>3</sup>

In December, 1911, Chicago was visited by an epidemic of sore throat, which was studied by Capps and Miller,<sup>4</sup> in relation to the milk supply. Wm. P. Coues,<sup>5</sup> made studies of an outbreak of sore throat infections which occurred in Boston in February, 1912, in which practically 45 per cent used milk from the same dairy. Besides the seven epidemics reported by Rosenau, W. S. Savage<sup>6</sup> has reported twelve milk-borne outbreaks in the British Isles and one in Christiania. All of these outbreaks occurred between 1880 and 1908.

It is very probable that outbreaks of sore throat other than the ones referred to, have occurred in this country, but on account of the difficulties attached to thorough epidemiological investigations they have been left to take care of themselves. Such studies, however, are very important, both to the general public and to physicians and investigators. Boards of Health and other authorities should spare no expense in encouraging such studies. The people are concerned because they must bear the brunt of the burden when epidemics occur and because they must carry out

\* Received for publication January 29, 1913.

<sup>1</sup> *Jour. Infect. Dis.*, 1911, 8, p. 259; *Boston Med. and Surg. Jour.*, 1911, 165, p. 899.

<sup>2</sup> *Bull. No. 12*, Mass. Board of Health, 1912, 7.

<sup>3</sup> *Bull. No. 56*, Hyg. Lab., U.S. Pub. Health and Mar.-Hosp. Serv., Wash., 1909.

<sup>4</sup> *Jour. Am. Med. Assn.*, 1912, 58, p. 1848.

<sup>5</sup> *Am. Jour. Pub. Health*, 1912, 2, p. 419.

<sup>6</sup> *Milk and the Public Health*, 1912.

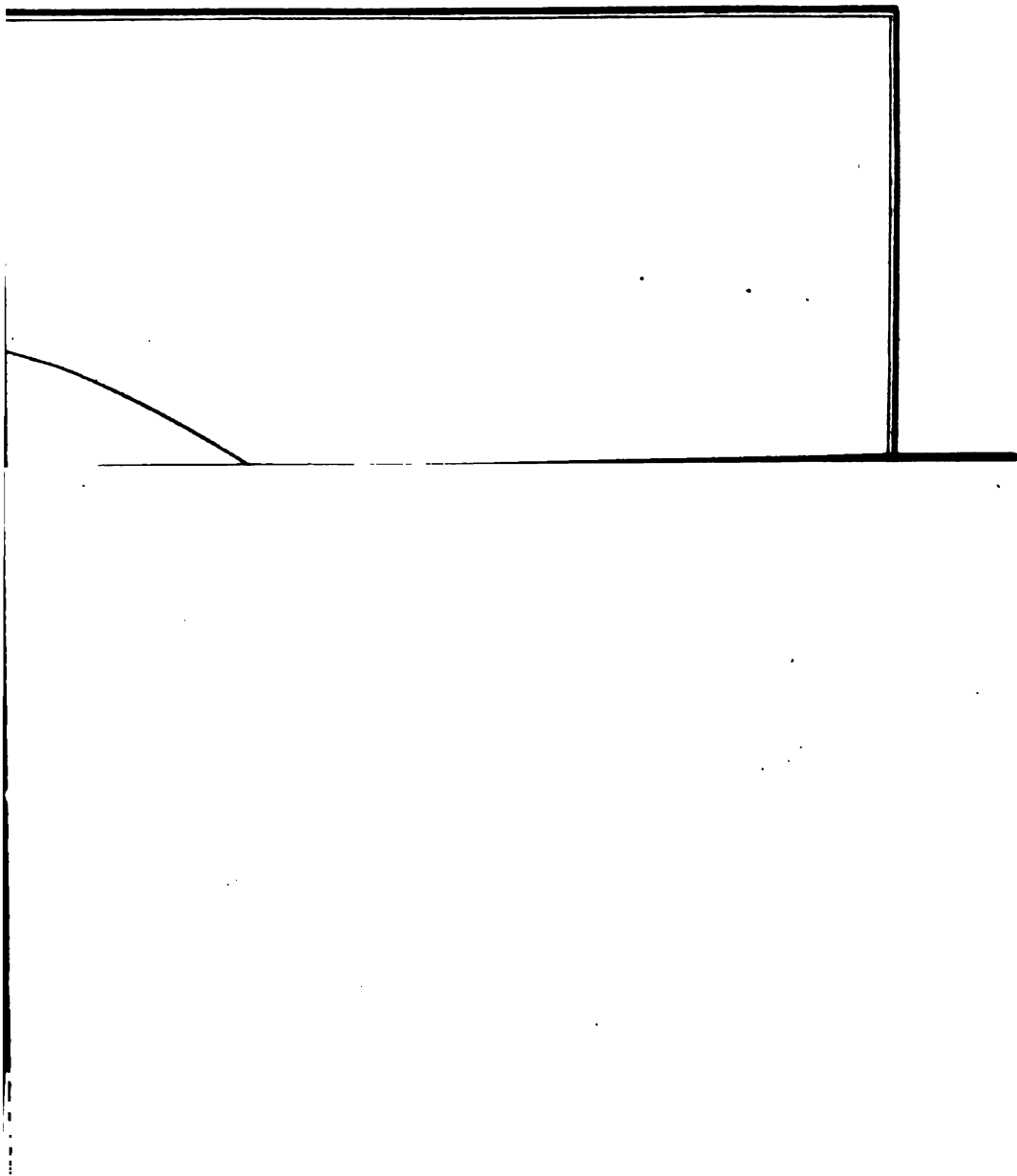
preventive measures. Medical men and scientific workers should be interested because such investigations may suggest the possibility of finding the transmitting agents, heretofore more or less obscure, of certain diseases occurring in epidemic form. As medicine develops more and more along rational lines, we can see more clearly how to proceed in studying measures of prevention.

It has not been very long since malarial fever was thought to be transmitted through the miasm of marshy, damp regions. Malarial districts were looked upon as made by nature and the prevention of the disease, except by the use of quinine, was not dreamed of. Now we know that the atmosphere has nothing to do with malaria, and marshy regions are concerned only so far as they afford breeding places for the *Anopheles* mosquito. As with malarial fever, so it may be with the transmission of most other diseases heretofore attributed to the air.

The study of the recent epidemic of sore throat in Concord impressed upon the writer how deep-rooted in the public is the idea that such outbreaks are due to atmospheric conditions. Even medical men are too often satisfied with this explanation, thus encouraging a public indifference that is akin to fatalism. It is reasonable to suppose that a room, carpeted and filled with upholstered furniture and objects that can easily catch dust, may become a source of danger in the presence of any disease, the causative agent of which is thrown off through the mouth and nasal passages. It is hardly probable, however, that any disease organism so thrown off can pervade the whole atmosphere and cause an epidemic.

The study of the outbreak of sore throat in Concord in January, 1912, was made, however, with a view of the following possible causative agents of transmission: air, water, contact, and food.

*Air.*—Concord, the capital city of New Hampshire, has 22,000 inhabitants. There are not many foreigners and a composite picture of the citizens would be much above the average. The outbreak began rather suddenly about January 1. Although it is said to have been very dusty before this date, the snow covered the ground January 4. Sleighing began at this time and continued





until March. Throughout this period there was practically no dust flying in the air. The disease was generally distributed throughout the city as shown in Map 1. It also prevailed at St. Paul's School, which is situated about two miles from the city limits. In the State Hospital for Insane, with over 950 inmates, not one was affected with sore throat. At Margaret Pillsbury Hospital the writer was told by the head nurse that during January practically every person, including both patients and attendants, was more or less affected with sore throat. At the Woman's Memorial Hospital, situated in the same part of the city, not a single case occurred, except one that was brought there as a patient. The matron told the writer, however, that when the case was admitted she was very uneasy and feared a general outbreak similar to the one at Margaret Pillsbury Hospital. At St. Mary's School, 25 cases among 50 persons occurred between January 12 and 28. At Rolfe and Rumford Asylum for children no cases occurred among 20 inmates. In the face of this evidence it seems that some medium other than the air was at fault in the transmission of the epidemic, or some of the 950 inmates of the State Hospital for Insane would probably have been affected. Some institutions would not have suffered so severely while others of like nature remained entirely free from the disease, since all were situated in the same part of the city.

*Water.*—The same facts that seem to eliminate the air can also be applied to eliminate the drinking-water. All of the above named institutions except St. Paul's used the same water supply. Records of the analyses also showed good water.

*Contact.*—The sharp outbreak of the disease as shown in Chart No. 1 indicates that some other medium of transmission was at work than the casual contact of one person with another. At St. Mary's school the girls came back from the holidays on the 8th. They began to get sick on the 12th without having come in contact, so far as they knew, with the disease. In Margaret Pillsbury Hospital, patients who had been in bed for weeks with some other trouble, suddenly became sick with sore throat. At St. Paul's School the boys returned from all parts of the country on January 10. In the different schools, Upper, Main, and Lower, they began to get sick

about the same time without having come in contact with one another.

Another interesting fact was the comparative freedom from the disease among the public-school children. There was ample opportunity for the disease to spread by contact among them, for several

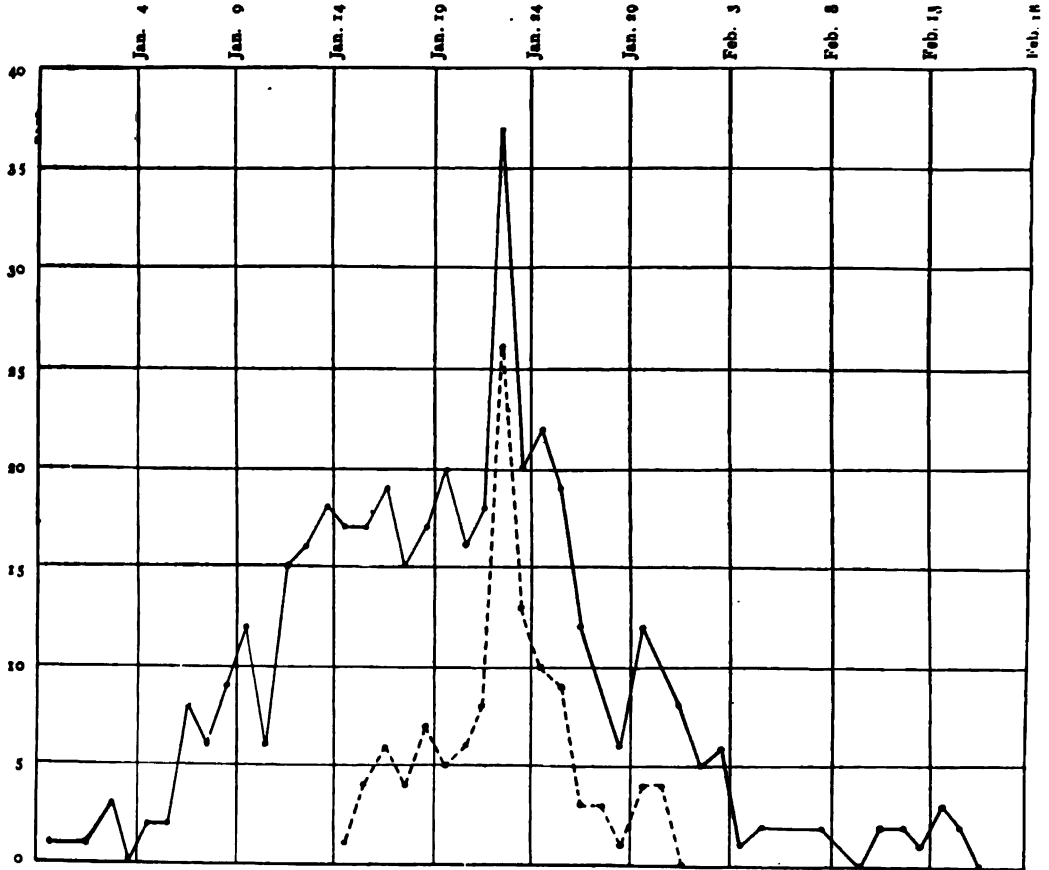


CHART 1.—Showing outbreak of 426 cases of "septic sore throat" in Concord, N.H., January and February, 1912. Dotted lines indicate outbreak at St. Paul's School.

admitted to the writer that they attended school with the disease. These cases had no physicians and are not included in the general record, but from the description given it is safe to conclude that they were affected with the "septic sore throat" that prevailed at the time.

If contact had been the means of the spread, why did it affect St. Paul's School and also St. Mary's where fifty persons were attacked, and not the different schools of the city where a few were known to have attended with the disease? These complications in connection with the rather sharp outbreak which died out in about six weeks, point to some other cause of transmission.

TABLE 1.  
NUMBER OF CASES REPORTED DAILY.

Date	Number Reported	Date	Number Reported	Date	Number Reported
Dec. 30, 1911.....	1	Jan. 16.....	14	Feb. 1.....	9
Jan. 1, 1912.....	1	" 17.....	16	" 2.....	3
" 2.....	1	" 18.....	16	" 3.....	6
" 3.....	4	" 19.....	14	" 4.....	1
" 4.....	0	" 20.....	21	" 5.....	2
" 5.....	2	" 21.....	15	" 6.....	2
" 6.....	3	" 22.....	17	" 7.....	2
" 7.....	8	" 23.....	36	" 8.....	1
" 8.....	6	" 24.....	19	" 9.....	1
" 9.....	9	" 25.....	21	" 10.....	0
" 10.....	15	" 26.....	18	" 11.....	1
" 11.....	6	" 27.....	12	" 12.....	2
" 12.....	12	" 28.....	7	" 13.....	1
" 13.....	15	" 29.....	6	" 14.....	3
" 14.....	16	" 30.....	10	" 15.....	1
" 15.....	16	" 31.....	8		

*Food.*—Investigating the food, it was soon noticed that there was a relation between the incidence of the disease and the use of cream and milk from one dairy. Inquiry in relation to foods other than milk and cream showed nothing. At St. Paul's School where 121 cases occurred, and at Margaret Pillsbury Hospital where 100 per cent were more or less affected, no connection with any particular food except the milk and cream was discovered. At first even the milk-supply did not seem to be involved. The writer was told that St. Paul's had its own model dairy. He was told that Margaret Pillsbury Hospital received its milk from one dairy and St. Mary's from another. Individuals in the city gave the same information when asked concerning their milk. Inquiry regarding the cream, however, showed a very suspicious relation to the outbreak. So generally distributed was the disease among the different milk routes, that the local authorities had almost decided that the milk was not at fault. Newspaper articles appeared, stating that the milk was not the cause. Upon further inquiry, it



was found that St. Paul's School, besides its regular home-produced milk, used cream from a dealer who supplied daily about 150 quarts of cream and 300 quarts of milk to the city of Concord. The Margaret Pillsbury Hospital used the same cream and St. Mary's School used this dealer's milk. Persons in the city who had been attacked also used this cream. Suspicion that the cream and milk from the dairy in question were chiefly responsible for the transmission of the epidemic, was strengthened when it was found that the State Hospital for the Insane, the Woman's Memorial and Rolfe and Rumford Asylum, in which institutions no cases developed, did not use either milk or cream from the suspected dairy. Upon this evidence the writer decided to make a more thorough investigation of the milk and cream supply in relation to the outbreak of sore throat.

TABLE 2.  
CREAM SUPPLY TO ST. PAUL'S SCHOOL.

Date	Upper	Main	Lower	Remarks
January 11.....	10 qts.	8 qts.	4 qts.	Upper school for large boys. Main school for middle boys. Lower school for small boys.
January 15.....	5 "	.. "	4 "	For ice cream.
January 17.....	.. "	4 "	.. "	For ice cream.
January 20.....	5 "	4 "	4 "	For cream dessert.
January 22.....	5 "	.. "	4 "	For ice cream.
January 24.....	.. "	4 "	.. "	For ice cream.
January 27.....	10 "	8 "	4 "	For cream dessert.

CREAM SUPPLY FURNISHED BY THE BOYS.

January 13.....	16 "	8 "	20 "	Cream supplied by boys used mostly on cereals.
January 20.....	16 "	16 "	20 "	
January 27.....	14 "	14 "	20 "	Three quarts were delivered daily to the Upper School.

The epidemic as shown in Chart 1 lasted from January 1 to February 15. That a few cases appeared after this date is not surprising, when we take into consideration the fact that nothing was done to cut off the source of trouble. A study of conditions will show why a gradual rather than a rapid decline in the incidence curve would be expected. Dairy conditions around Concord were such that it was not unlikely that other supplies could easily have become involved after so many persons had had the disease.

It stands to reason that as soon as the cases on the farms supplying the suspected dairy and the cases at the suspected dairy itself, convalesced, the outbreak would die down. The outbreak at St. Paul's School seemed to be sharper than in the city. The figures of the number sick were kindly furnished by the school physician, Dr. Walker. Unfortunately the writer was not able to make inquiries among the students, regarding the number affected after January 29, who did not ask for medical attention. The writer's investigations in Concord began on February 12 and ended about March 15. The authorities in the school, because they did not wish to bring the subject before the boys again, preferred to have the matter dropped. Argument that the findings might be of great public interest availed nothing. The writer was thus unable to make as thorough a study in St. Paul's School as he wished. It is unfortunate that heads of many of our institutions of learning do not seem to realize the importance of more careful attention to the questions which affect the public health. The authorities, it seems, should have encouraged every effort to find out the cause of the disease that brought down 10, 15 and 20 boys a day. It is just such indifference that has caused the needless sacrifice of so many lives in this country.

Since the disease was not a reportable one, none of the cases had been reported to the local health department. In the absence of public records it was necessary to depend on the local physicians for all records. A card similar to the following was distributed among them.

No. ....  
 Name. .... Street. .... Age. .... Sex. ....  
 Occupation. .... Date when sickness began. ....  
 No. in family. .... How many in family were affected. ....  
 Interval in cases in same family. .... Butter supply. ....  
 Was water other than city water used? .... Cream supply. ....  
 Name of dairyman. .... Name of grocer. ....  
 Fruit supply. ....  
 Type and complications of disease. ....  
 ....  
 Name of Physician. ....

The physicians very kindly gave the name, address, age, sex, and date when sickness began. The cards were then called for by

the writer and the other data were obtained by direct communication with the families. In this way records of over 400 cases were secured. Unfortunately one or two physicians were not interested and would not give any data. One did not consider it giving his patients a "fair deal to furnish their names and addresses." Another was always "too busy" and informed the writer that he did not "take much stock in the cream theory, anyway." This was after a few days' pointed inquiry had naturally started rumors regarding the cream supply. With these exceptions the physicians were very co-operative. Their influence among the people was such that in no instance was the writer refused the desired information. It will be safe to say that the records in this paper cover 75 per cent of the cases that employed physicians. Although no figures are available to show the number of persons who did not seek medical attention, although suffering from the disease, there were probably several hundred. It is a conservative estimate to say that at least 1,000 persons in the city were attacked between January 1 and February 15.

*Dairy conditions.*—The dairy conditions of Concord, New Hampshire, compare favorably with conditions in Massachusetts except as regards the handling of the milk in the milk rooms. In Massachusetts the milk is handled in milk rooms removed from the kitchen. On the farms supplying Concord the milk is generally handled in the kitchen. The cans are usually washed in the kitchen. As a rule no means of sterilizing the cans exist. The barns are of the typical New England type, built for warmth at a sacrifice of light and fresh air. In some cases cement floors were found and these as a rule were fairly clean. In most cases, however, the floors were dirty and showed that the manure was not regularly removed. The cows were in some cases clean and showed that they were regularly curried. In others the manure was caked on their hind quarters.

The investigations on the farms were made in company with Dr. A. H. Rose of the Massachusetts State Board of Health, who was in Concord investigating for his Board as a safeguard against trouble in Massachusetts. The following evidence in regard to the disease on the farms has the advantage of having been taken by two persons. The amount of milk credited to the dairymen, and also to the dealers in another part of the paper, is only approximate.

# OUTBREAK OF SEPTIC SORE THROAT IN CONCORD, N.H. 489

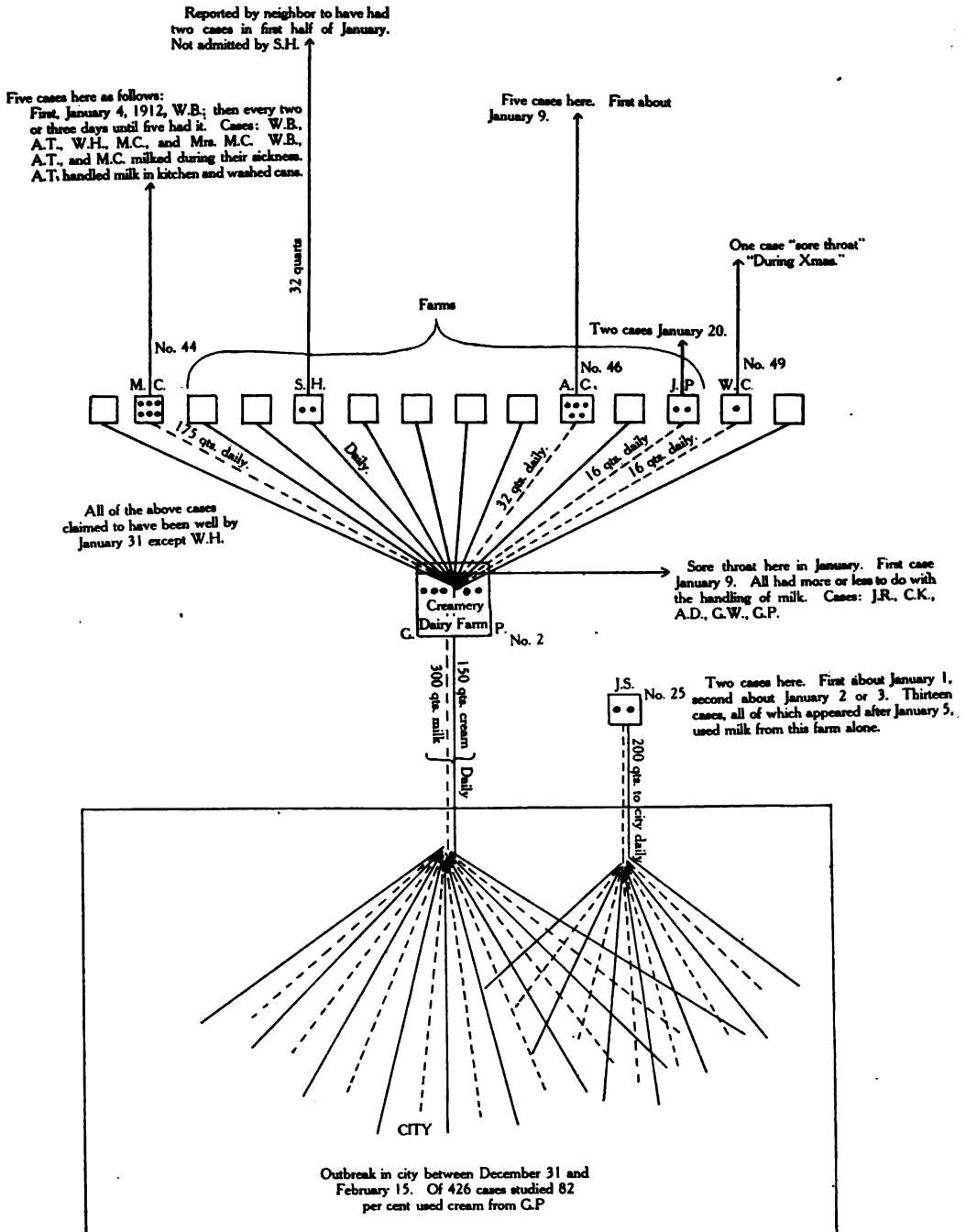


FIG. 1.—Showing the probable route of infection in 84 per cent of the cases. Dotted lines indicate route of infected milk and cream.

Dairy No. 25, J.W.S., retailer, 28 cows. Daily milk supply, 200 quarts. Mrs. J.W.S. was sick about January 1. "Bronchitis and sore glands in the neck." A little girl was also sick here about January 2 or 3. Milk cans were found in the kitchen giving evidence that they were washed there.

Dairy No. 37, J.F.H., wholesaler, 17 cows. Daily milk supply, 85 quarts, to Webber. At this place was found Mrs. M.A.S., who claimed to be getting over an attack of sore throat. The cervical glands were enlarged and there was a slight redness of the throat. She was taken sick February 8 while in the city and had boarded at a restaurant that had cream from the suspected dairy. Cans were found at the entrance of the kitchen. Mrs. M.A.S. was a visitor and claimed not to have anything to do with the milk.

Farm No. 45, S.A.H., wholesaler, 7 cows. Daily milk supply, 33 quarts. Milk sold to Putnam. About January 8, two young boys had sore throat at this farm. The cans were washed and the milk handled in the kitchen.

Farm No. 46, A.A.G., wholesaler, 5 cows. Daily milk supply, 22 quarts. Milk sold to Putnam. During January there were five cases of sore throat at this farm, four children and the father. The first case occurred about January 9, and the others appeared every day or two in succession.

Farm No. 44, M.C., wholesaler, 27 cows. Daily milk supply, 175 quarts. Milk sold to Putnam. The milk was handled on a shelf back of the cows and cooled in tubs in the yard. The cans were washed in the kitchen. There were five cases of sore throat here as follows: W.B., the first case, January 4; W.H., the second case, who was affected until the middle of February; A.T., the third case; M.C., the fourth case; Mrs. M.C., the fifth case. W.B., A.T., and M.C. milked the cows during their sickness. A.T. washed the cans and handled the milk in the kitchen.

Farm No. 49, W.G., wholesaler, 2 cows. Daily milk supply, 16 quarts, to Putnam. W.G. said: "I had tonsilitis about Christmas, but was not bothered much." He handled his own milk.

Farm No. 2, G.A.P., 32 cows. This dealer also bought milk from the surrounding country. His average daily supply in milk was 300 quarts; cream, 150 quarts. This dairy was well ventilated and the cows were apparently in good physical condition. A milk room, adjoining the kitchen, was fitted, however, with an independent heating outfit. There were large stationary wooden tubs for cleaning the bottles and cans. On one end of the tub stand was an iron pipe extending up about six inches. It was customary to invert cans over this and submit them to live steam. This was the only sterilizing apparatus found. In the milk room was a cream separator. On the day of the writer's visit the separator was clean from a standpoint of domestic cleanliness, but although the detachable parts had not been sterilized, they were placed ready for future use.

From the facts elicited in connection with this dairy there is no doubt that directly and indirectly it was responsible for much of the trouble in the city. The milk brought in from surrounding farms was separated and the cream was delivered to dealers in Concord. At several of these farms undoubted evidence of sore throat was found. Persons admitted having had the disease in January.

They admitted that other members of their families had had it and while sick had worked around the cows and milk. At G.A.P.'s dairy also there were five cases. The first started about January 9. One person that worked in this dairy admitted that his neck was quite sore, but said he did not think the trouble was serious and did not have a physician. Throughout his entire sickness he worked in the dairy separating the milk and washing the cans.

Only those dairies to which cases of sore throat could be traced, are included in this report. The conditions and methods of all the dairies examined were similar to the ones mentioned.

TABLE 3.  
PERCENTAGE OF MILK SUPPLIED BY DEALERS AND ALSO PERCENTAGE OF CASES ON EACH  
DEALER'S ROUTE.

NAME	PERCENTAGE		
	Amount of Milk	Including Putnam's Cream	Not Including Putnam's Cream
Abb.....	2.3	4.6	0.4
All.....	2.3	0.0	0.0
Ann.....	2.1	1.4	0.4
Bad.....	1.2	1.0	0.2
Bal.....	3.0	2.1	0.8
Buc.....	3.0	3.3	1.3
Bar.....	9.0	6.1	2.0
Bro.....	1.2	0.6	0.0
Cha.....	0.4	0.8	0.4
Cha.....	0.4	0.8	0.4
Che.....	0.5	1.0	0.4
Cie.....	2.8	1.0	0.8
Cla.....	0.5	0.0	0.0
Clo.....	2.1	0.8	0.4
Cha.....	0.4	0.8	0.4
Eme.....	1.1	0.2	0.0
Eas.....	2.3	2.3	0.0
Fie.....	6.1	4.2	1.6
Faw.....	2.3	4.4	0.8
Hig.....	0.8	0.0	0.0
Hil.....	3.0	2.1	0.2
Hil.....	0.5	5.8	0.2
Jon.....	1.0	0.2	0.0
Jor.....	2.3	6.7	0.0
Kim.....	2.0	1.4	0.0
Lam.....	0.1	0.0	0.0
May.....	2.2	1.6	0.0
Pot.....	2.3	0.4	0.0
Pot.....	1.8	0.4	0.0
Put.....	14.0	81.0	81.0
San.....	1.8	0.4	0.4
San.....	2.3	7.8	2.9
Sar.....	1.8	0.8	0.4
Sim.....	3.0	2.7	0.4
Spo.....	1.3	1.0	0.4
Ten.....	2.8	0.6	0.0
Ven.....	1.4	0.4	0.2
Web.....	4.1	3.0	1.2
Whi.....	1.4	0.2	0.2
Unknown.....	10.0	1.0	1.0

TABLE 4.  
FIFTY FAMILIES STUDIED IN RELATION TO THE DISEASE AND THE MILK SUPPLY.

Number of Family	Number in Family	Number Sick	Milk Supply	Cream Supply	Remarks
1.....	7	1 (?)	C. Jones	.....	Mr. A says he had sore throat one day in January, but had no physician.
2.....	4	0	Barnard & Dunn	.....	Cases in March. Mrs. B. says she had a slight sore throat in January, but had no doctor.
3.....	3	2	Badger	Putnam	
4.....	5	1 (?)	Own milk and cream	.....	
5.....	4	1	Abbott	Putnam	This is a boarding-house.
6.....	3	0	Own milk and cream	.....	
7.....	15	3	.....	Putnam now and then	
8.....	3	0	Pettimore (one cow)	.....	Patient ate at restaurants also before sickness.
9.....	4	0	Cilley	.....	
10.....	4	0	Cilley	.....	
11.....	3	1	Barnard & Dunn	Whittier	
12.....	4	0	Field	.....	Putnam now and then
13.....	5	0	C. Potter	.....	
14.....	8	0	Clough	.....	
15.....	4	2	C. Jones	Putnam now and then	
16.....	4	0	Fowler	.....	Webber; occasionally from Putnam
17.....	2	0	Webber	.....	
18.....	4	0	Fowler	.....	
19.....	2	0	Own milk	.....	Putnam
20.....	2	2	Fowler	.....	
21.....	2	1	Webber	Putnam	
22.....	3	0	Own milk and cream	.....	Putnam
23.....	5	0	Hillman	.....	
24.....	2	1	Putnam	.....	
25.....	2	1	J. W. Sanborn	Putnam once a week	
26.....	5	0	Tenney	Putnam once or twice during January	
29.....	4	1	Fowler	Putnam	Putnam once a week
30.....	4	0	Fowler, also Fields	.....	
31.....	2	0	J. W. Sanborn	Page	
32.....	4	0	Own milk and cream	.....	Putnam once a week
33.....	5	0	Cilley	.....	
34.....	3	0	J. W. Abbott	.....	
35.....	4	0	Own milk and cream	.....	Putnam
36.....	3	0	Cilley	.....	
37.....	2	0	Cilley	.....	
38.....	4	0	J. W. Sanborn	Putnam	Putnam
39.....	3	0	Sargent	.....	
40.....	3	0	Barnard & Dunn	.....	
41.....	3	3	Brown	Putnam	Mrs. Hill Chamberlain
42.....	3	0	Buckley	.....	
43.....	6	0	Kimball	.....	
44.....	3	0	Fowler	.....	Patient, an engineer ate away from home very often.
45.....	3	1	Worcester	.....	
46.....	2	0	Fowler	.....	
47.....	1	0	Tarleton	.....	Boarding-house.
48.....	20	0	Abbott, also Field	Putnam's cream now and then	
49.....	5	0	Annis	Putnam sometimes	
50.....	5	2	W. H. Chamberlain	.....	Mr. C. ate at restaurant. Child had it after father recovered.

This table corresponds to Fig. 2.

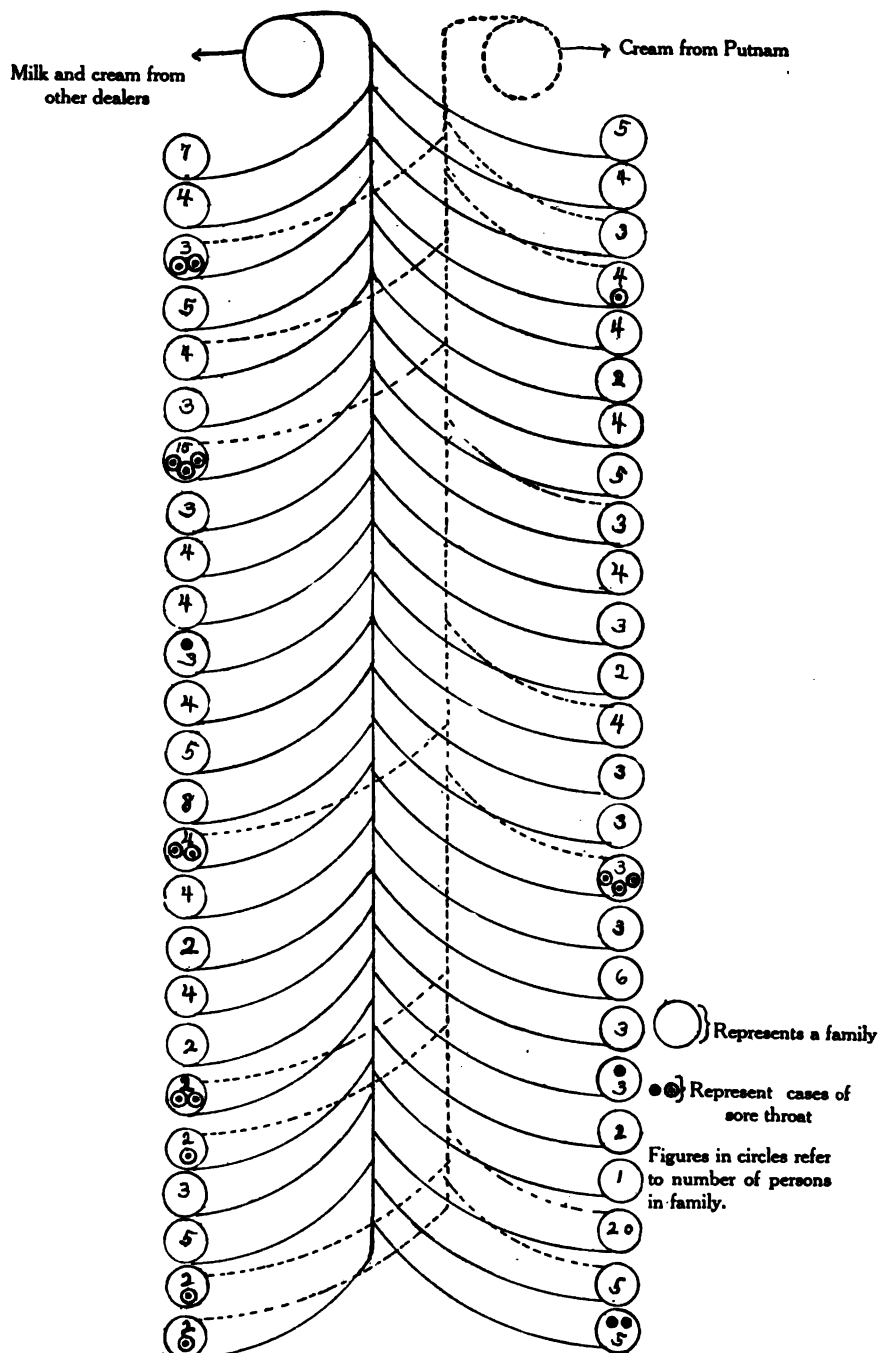


FIG. 2.—A graphic representation of 50 families taken at random in the city showing a close relation between the cream supply and the disease.



*The disease.*—The disease corresponds to the description found in the text books on pseudodiphtheria; Holt classifies under this head, all inflammations of the throat and upper air passage characterized by the production of a false membrane in which the Klebs-Loeffer bacillus is not found. The term "septic sore throat" seems to be a good name for the disease and is now generally used.

*Etiology.*—The outbreak was general throughout the city. Eighty-two per cent of the cases occurred among the users of milk and cream from one dairy. It is probable that some cases classified with other dealers also used cream from this dairy since ice cream and milk served in a few drug stores came from the suspected dealer. Unfortunately the writer could not get records bearing on the relation of ice cream and the disease, since most of the families that used ice cream also used the raw cream. In one family, where four were attacked practically at the same time, cream from the suspected dairy had been purchased a day or two before the sickness and was put in the sherbet. Particular attention was paid to this family, and the housewife stated that not all of the sherbet had been used when the first person was taken ill. The disease as a rule affected the better classes, which is interesting because it shows the relation to the users of cream. In one apparently very poor family, where four out of six were attacked about the same time, the milk supply was from one single cow. When questioned about buying cream, they said they "never bought cream." Upon further inquiry it was found that the mother worked where cream and milk from the suspected dairy was used, and that she habitually brought cream and milk to the children and to the bed-ridden grandmother. The two not affected, one a man and the other a nursing child, did not use any milk or cream.

The disease was probably due to a form of streptococcus. Unfortunately no exhaustive study of the organism causing the disease was made. The writer's investigations began too late to enable him to make a satisfactory bacteriological study of the trouble. Dr. Duncan, state bacteriologist for New Hampshire, examined more than 100 cultures. He told the writer that in his opinion the disease was due to a streptococcus. His observations were made in a routine examination of swabs sent into the labora-

tory for diagnosis. Smears from two cases were examined by the writer and a streptococcus morphologically similar to the one described by Davis,<sup>1</sup> in the Chicago outbreak, was found.

The descriptions of the disease as furnished by the physicians

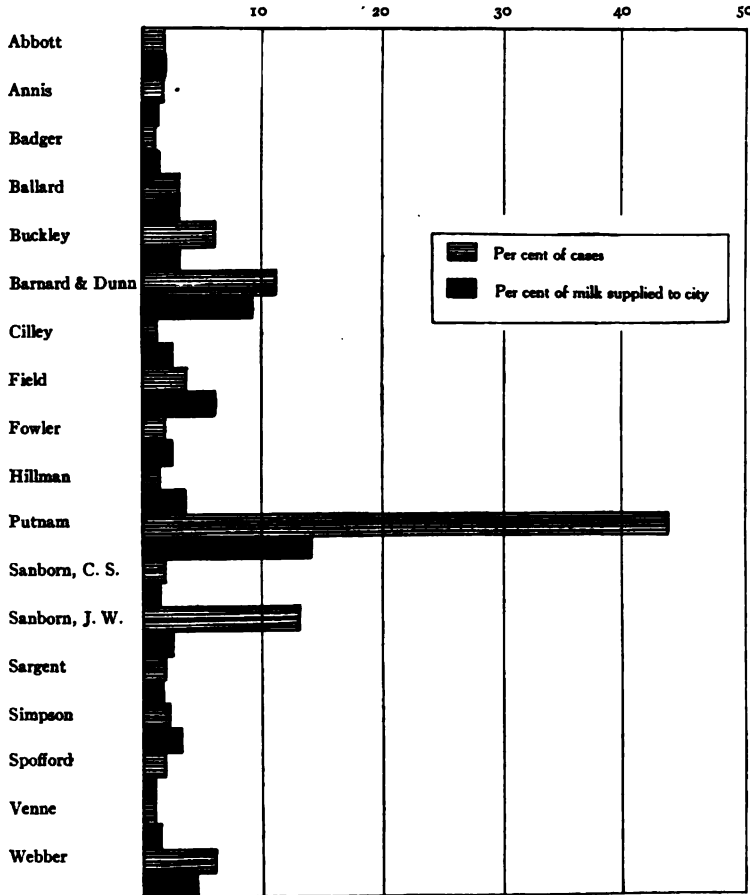


CHART 2.—Showing distribution of 110 cases that used a single milk supply exclusively.

of Concord, for the most part, were similar and were about as follows: "Follicular tonsilitis with marked swelling of the cervical glands. The tonsils are highly inflamed with dirty whitish dots over the crypts. Then follows the swelling of the cervical glands.

<sup>1</sup> *Jour. Am. Med. Assn.*, 1912, 58, p. 1852.

Suppuration may follow." "In nearly all, first symptoms, tenderness of glands, submaxillary and postcervical. Swelling of the same. Then comes complaint of difficulty of swallowing, sore throat, pharynx red and swollen; tonsils also. Relapses were common and in cases persistent. Many cases better in a few days, then relapsed." "Fever—'Grippe pains' in about 25 per cent of the cases. Tonsillitis in about 50 per cent. Enlarged submaxillary glands in 50 per cent." Four deaths were recorded.

The summary of answers by ten physicians regarding complications showed the following:

Cases complicated with erysipelas.....	7
“ “ “ pneumonia.....	1
“ “ “ tonsillar abscess.....	6
“ “ “ external abscess.....	4
“ “ “ otitis media.....	8

This probably includes about 300 cases.

*Age.*—The relation of age shows that young children were not affected as much as grown persons. Of 249 cases not including the boys and girls at St. Paul's and St. Mary's, the ages were as follows:

Under five.....	20
Between five and twenty.....	24
Between twenty and thirty.....	104
Between thirty and fifty.....	55
Over fifty.....	46

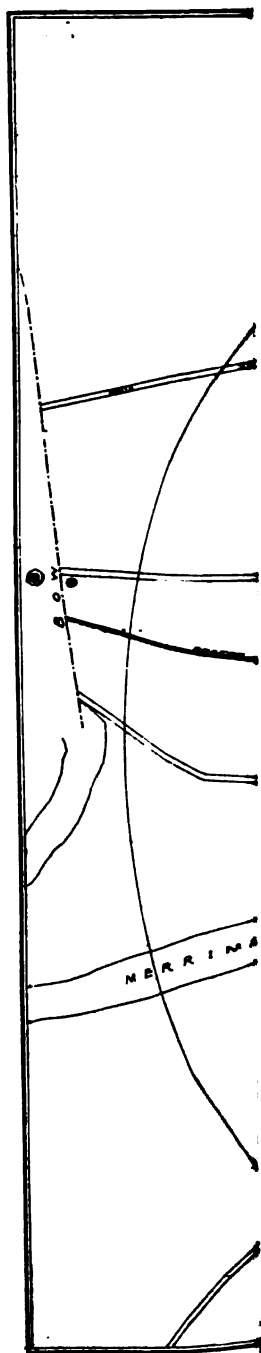
The children attacked, however, gave history in most cases of using the suspected cream. The cream was used generally in two ways, whipped for desserts, and on cereals.

TABLE 5.  
NUMBER OF PERSONS IN FAMILY AND NUMBER AFFECTED IN EACH.

No. in Family	No. of Families	No. Sick
2	19	24
3	34	60
4	27	47
5	17	49
6	20	57
7	3	8
11	1	2

Average number in each family sick, 2.

Average number in each family, 3.5.





*Sex.*—Of 427 cases studied in regard to sex including 128 boys at St. Paul's and 25 girls at St. Mary's Schools, there were 240 males and 187 females.

Not including these schools there were males, 119; and females, 162.

#### CONCLUSIONS.

The epidemic of "septic sore throat" in Concord, New Hampshire, in January, 1912, was spread by means of infected *cream and milk*: the milk became infected through the handling by persons suffering with the disease, and the cream when separated from the milk retained the infecting organisms.

The standards of intelligence among milk producers are too low, and the public should demand that the producers have an elementary knowledge of bacteriology, or at least be able to recognize the importance of safeguarding milk against bacterial contamination.

Under the present standards, it is not safe to use market milk in the raw state and efficient pasteurization should be insisted upon in all cases where vending is engaged in.

It is just as important to safeguard the cream supply as the milk supply.

I wish to thank Dr. M. J. Rosenau of Harvard, for making it possible for me to undertake this epidemiological study; also the trustees of the Bach fund for an appropriation of \$100 for scientific research, and Dr. A. H. Rose, dairy inspector for Massachusetts, whom I accompanied on many trips to dairy farms around Concord, for his advice and aid.

I wish also to thank the local and state health departments of Concord, New Hampshire, for allowing the use of their offices, and the physicians of Concord, whose co-operation made it possible to gather the necessary data. To Dr. B. F. Arms and A. I. Kendall I express appreciation for laboratory aid.



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